STRUCTURAL CHARACTERIZATION OF CARBOHYDRATE ATTACHED TO THE GLYCOPROTEIN CELLULASE ENZYMES OF *Trichoderma reesei* QM 9414

By

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I wish to dedicate this work first to my wife, Linda, who stood by my side and who provided encouragement, love and emotional support throughout and also to my two sons, Colin and Ian, who were both born during this time.

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ABBREVIATIONS

CBH I(D)	-	Cellobiohydrolase I(D) from T. reesei QM 9414
CBH II	-	Cellobiohydrolase II from T. reesei QM 9414
GLC	-	Gas-liquid chromatography
GC/MS	-	Gas chromatography/mass spectrometry
GC/MS/DS	-	Gas chromatograph/mass spectrometer/data system
EI	-	Electron impact
CI^+	-	Chemical ionization
M^+	-	Molecular ion
NMR	-	Nuclear magnetic resonance
HPLC	-	High pressure (performance) liquid chromatography
PAA	-	Peracetylated alditol
PAAN	-	Peracetylated aldononitrile
DEAE	-	Diethylaminoethyl
SP	-	Sulfopropyl
Ser	-	Serine
Thr	-	Threonine
Asn	-	Asparagine
Abu	-	α-aminobutyric acid
Xyl	-	Xylose
Man	-	Mannose
Glc	-	Glucose
GlcNAc	-	N-acetylglucosamine
M, M_2, M_2G, M_3G	-	Oligosaccharides released from the cellobiohydrolases (where M = Man and G = Glc)
DMSO	-	Dimethylsulfoxide
TSP	-	(Trimethylsilane)-1-propane sulfonate

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Chairman: Dr. Ross D. Brown, Jr.
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Cellobiohydrolases I(D) and II were purified by ion exchange chromatography from an extracellular culture filtrate of *Trichoderma reesei* QM 9414. Neutral sugar composition of each was determined by gas-liquid chromatographic analysis of the peracetylated alditol and aldononitrile acetate derivatives of sugars released by either reductive ß-elimination or acid hydrolysis. Mannose and glucose were found to be the only neutral sugars and were covalently attached to the proteins through alkali-labile mannosyl residues. Analysis of the alkaline borohydride-released carbohydrate by high pressure liquid chromatography (HPLC) demonstrated that each molecule of cellobiohydrolase I(D) contained 5.9% carbohydrate comprising 0.7 tetra-, 4.2 tri-, 1.1 di- and 1.2 monosaccharides and that cellobiohydrolase II contained 18.9% carbohydrate comprising 14.9 tri-, 1.6 di- and 9.0 monosaccharides. The purified oligosaccharides were shown by methylation analysis to contain (1-2) and (1-6)

glycosidic linkages and the position of 6-substituted residues was confirmed by acetolysis. The sequence and anomeric nature of the sugar residues in each oligosaccharide was determined by sequential glycosidase digestion and all the residues were found to be α-linked. Proton decoupled ^{13}C-NMR analysis suggested that for cellobiohydrolase I(D), each oligosaccharide was attached to a threonyl residue on the polypeptide and for cellobiohydrolase II, each oligosaccharide was attached to threonyl and seryl residues on the polypeptide. These data were supported by amino acid analysis for α-aminobutyric acid after β-elimination and palladium chloride treatment of each protein. Coupled ^{13}C-NMR analysis confirmed that the anomeric carbon of each sugar residue was in the α-configuration and ^{1}H-NMR identified the presence of mannosyl and α-glucosyl residues in the purified oligosaccharides. The structures of the oligosaccharides attached to the enzymes were determined to be mannosylα(1-2)glucosylα(1-6)mannosylα(1-2)mannose, glucosylα(1-6) mannosylα(1-2)mannose, mannosylα(1-2)mannose and mannnose. This is the first report of a unique glucosylα(1-6)mannose linkage. Thus, work with the cellobiohydrolases and the endoglucanases indicates that the predominant cellulase enzymes secreted by T. reesei QM 9414 are each glycosylated with similar oligosaccharides.

...

INTRODUCTION

Ghose [1] has estimated the annual worldwide production of biodegradable substances through photosynthesis at approximately 1.8 x 10^{12} tons, of which 40% is cellulose. As much as 25% of this [2] could be made available for conversion processes, a significant amount of which occurs as agricultural and municipal wastes. Therefore, in the future, cellulose must be regarded as an important potential source of fuel, food and chemical feedstocks.

Enzymatic saccharification of cellulose has proved to be both specific and efficient, and among the organisms tested, Trichoderma species showed the highest levels of extracellular activity [3]. Most fungal cellulases have been shown to contain covalently attached carbohydrate, although the complete structure of the oligosaccharides attached to the polypeptide of a cellulase enzyme has never been elucidated.

Glycoproteins

Proteins which have covalently associated carbohydrate are termed glycoproteins [4]. These are a diverse group of macromolecules found throughout nature in plants, animals and microorganisms. Glycoproteins are implicated in a wide variety of functions including cell adhesion, molecular recognition, structural support, lubrication,

hormone control, blood clotting and enzyme catalysis. With the increased sophistication of modern analytical methods, the structure of the oligosaccharides attached to many glycoproteins have been determined and are well documented [4-8]. The oligosaccharide side chains of glycoproteins fall into two general classes, (i) those which are attached via an N-glycosidic linkage from an N-acetylglucosaminyl residue to the amide nitrogen of an asparaginyl residue on the protein and (ii) those which are O-glycosidically linked from a neutral sugar or an N-acetylgalactosaminyl residue to either a seryl, threonyl, hydroxyprolyl or hydroxylysyl residue on the protein.

The N-linked oligosaccharides are similar in that they all contain a pentasaccharide core structure, Manα(1-3)[Manα(1-6)]Man-ß(1-4)GlcNAcß(1-4)GlcNAcß-(Asn), attached to the protein. This class of oligosaccharides comprises three general groups, (i) those that have only mannosyl residues in addition to the core are termed "simple", (ii) those that have one or more of a variety of sugars, galactose, N-acetylgalactosamine, sialic acid and/or fucose in addition to the core, are termed "complex" and (iii) those that have a mixture of "simple" and "complex" are termed "hybrid". The N-linked class of glycoproteins has a specific amino acid sequence requirement for addition of the carbohydrate to the protein. The sequence Asn-X-Thr(Ser) must be present, where X can be almost any amino acid except proline [5].

Oligosaccharides of this class are transferred from a lipid-linked precursor *en bloc* to the protein. This precursor is a large branched structure containing mannose and glucose. Following transfer, the glucosyl residues and most of the mannosyl residues are removed

and the core is subsequently enlarged to the simple, complex or hybrid forms. The glycoproteins containing these types of oligosaccharides are represented diversely in nature. They are cell surface [9,10], secretory [11,12], plasma [13,14] and hormone polypeptides [15,16] to mention a few.

The O-linked oligosaccharides are not known to have such an amino acid sequence requirement, although it has been suggested that a conformational prerequisite, i.e. a ß-turn, is necessary. Sugars are thought to be added directly to the protein one by one rather than by transfer of an oligosaccharide *en bloc*. This class of oligosaccharides also comprises three general groups, (i) those that have N-acetylgalactosamine attached to the hydroxyl group of serine or threonine, (ii) those that have neutral sugars attached to the hydroxyl group of hydroxylysine or hydroxyproline and (iii) those that have neutral sugars attached to the hydroxyl group of serine or threonine. The oligosaccharides of the first group are generally short, one to six residues in length, although some are much larger. They may also contain other sugars such as galactose, fucose, N-acetylglucosamine and/or sialic acid on the chains. This group is exemplified by the mucins, but also includes antifreeze glycoproteins [17], chorionic gonadotropin [18], blood group substances [19] and cartilage keratan sulfate [20]. The second class is characterized by several structural proteins, the collagens [21,22] and basement membrane proteins [23,24] in animals, which have galactose O-linked to hydroxylysine and a group of plant glycoproteins called extensins, which have arabinose and galactose attached to hydroxyproline. For the third group, the neutral sugar can be xylose in the case of

chondroitin sulfate and dermatan sulfate [25], galactose in the case of cuticle collagen [26,27], fucose in the case of several mammalian cell line glycoproteins [28,29] and mannose in the case of fungal [30-38] and yeast [39,40] glycoproteins.

Fungal Glycoenzymes

With the exception of cuticle collagen from Nereis virens, which was found by Spiro and Bhoyroo [26] to contain a unique acidic disaccharide of 6-O-α-D-glucuronosyl-O-D-mannose, all of the glycoproteins currently known to contain a mannosyl-O-Thr(Ser) linkage are of fungal origin. Of these, the majority are fungal carbohydrases which are secreted from the respective organisms.

The glucoamylases ((1,4)(1,6)-α-D-glucanglucohydrolases) from a number of Aspergillus species have been analyzed for carbohydrate content and composition [31-35]. Pazur et al. [31] removed the neutral carbohydrate of glucoamylase I from A. niger with mild alkaline treatment. The oligosaccharide chains released ranged in size from one to five sugars, with 20 monosaccharides, all being mannose; 11 disaccharides, all mannobiose; and the larger chains which were combinations of glucose, galactose and mannose. Methylation and G.C./M.S. analysis determined the disaccharides to be (1-2) linked and the larger species to be branched and containing (1-3) and (1-6) glycosidic bonds. The structures of these larger species, however, were never definitively described. Manjunath and Rao [33,34] prepared a series of glycopeptides from glucoamylase II of A. niger following extensive pronase digestion. Monosaccharides were subsequently

released from the glycopeptides by sequential glycosidase digestion and the products separated and identified by paper chromatography. A series of trisaccharides were described with the general formula, X-Man-Man-, where X was either glucose, galactose, mannose, N-acetyl-glucosamine or xylose. The residues were α-linked in each case. The procedure used, however, did not exclude the possibility of branched structures, as substitution of individual sugars was not determined by classical methylation analysis. Carbohydrate and amino acid analysis following reductive ß-elimination identified mannose as the only sugar attached to either threonine or serine on the polypeptide. Comparison of glucoamylases I and II from A. niger 1,2 and 3, Aspergillus foetidus and Aspergillus candidus showed that most of the enzymes contained mannose, glucose, galactose, xylose and glucosamine [35]. The Aspergillus glucoamylases are heavily glycosylated and contain between 48 and 151 monosaccharides covalently attached to each protein molecule [35,36]

Rosenthal and Nordin [37] have characterized a mycodextranase (endo-1,4-α-D-glucanase) from Penicillium melinii. Glycosidase digestions, Smith degradation and methylation analysis indicate that the oligosaccharides are present as about 25 mannose, Glcα(1-2)Man and Manα(1-2)Glcα(1-2)Man side chains.

Raizada and Schutzbach [38] characterized a Man α(1-2)Manα(1-2)Mannitol oligosaccaharide from a major cell envelope glycoprotein of the fungus imperfectus, Cryptococcus laurentii. The trisaccharide was removed from the protein by reductive ß-elimination using ^{3}H-$NaBH_4$ and the radiolabelled product isolated by gel

filtration. Characterization was performed by glycosidase digestion and comparison of the products with standards by paper chromatography.

While not enzymatic in nature, Saccharomyces cerevisiae cell wall mannan was shown by Nakajima and Ballou [39] to contain a number of similar O-linked mannooligosaccharides. These were released from the protein by mild alkaline treatment and individually purified by gel filtration. Glycosidase digestion and gas chromatography were used to identify a series of related oligosaccharides, the largest being Manα(1-3)Manα(1-2)Manα(1-2)Man.

Cellulases

Most cellulases are of fungal origin and are glycoprotein in nature, but relatively little is known about their structure. Unlike their α-glucanase counterparts such as the glucoamylases and the dextranases, the cellulases are thought to exist as a system of enzymes.

The cellulase system of Trichoderma reesei QM 9414 comprises three types of enzymes which act synergistically and can effect the complete conversion of both amorphous and crystalline cellulose to glucose [41,42]. The system consists of several endo-1,4-ß-Dglucanases (EC 3.2.1.4), two exo-1,4-ß-D-cellobiohydrolases (EC 3.2.1.91) and one or more ß-D-glucosidases (EC 3.2.1.21)[43,44]. Endoglucanases release soluble oligosaccharides and free chain ends from cellulose, cellobiohydrolases release cellobiose from free chain ends and ß-glucosidases release glucose from soluble cellooligosaccharides.

It has been shown that the biosynthesis of endoglucanases and cellobiohydrolases can be induced by addition of sophorose (O-β-D-glucopyranosyl-1,2-O-glucopyranose) to resting cells of this organism [43,45-47]. Gritzali and Brown [43] have shown that cellobiohydrolase I, form D (CBH I(D)) from cellulose-grown and sophorose-induced Trichoderma cultures have the same electrophoretic mobility, amino acid composition and total carbohydrate content suggesting that they are the same protein. CBH I(D) is so named as it has been shown to have the same amino acid composition as three other forms of cellobiohydrolase I, A, B and C from Trichoderma viride [43,48]. Gum and Brown [48] proposed that these forms only differ structurally in the amount of covalently attached carbohydrate. Cellobiohydrolase II (CBH II) from cellulose-grown cells also exhibited the same electrophoretic pattern as its induced counterpart although no comparative compositional studies on the induced enzyme were performed. CBH I(D) and CBH II have been shown by several criteria to be separate proteins. Apart from electrophoretic mobility, the two have distinct amino acid compositions [42,49]. Fägerstam and Pettersson [42] have also determined that the sequence of the N-terminal 20 amino acids for each polypeptide was different, although both N-terminii were blocked by a pyroglutamic acid residue.

Most of the cellulolytic enzymes isolated from Trichoderma are glycoprotein in nature [30,43,48-56], although some have no covalently bound carbohydrate [57,58]. The carbohydrate content of each type of cellulase from this genus varies considerably and is summarized in Table I. Gum and Brown [30] have presented the only attempt so far at structural characterization of the carbohydrate of a cellulolytic

TABLE I

CARBOHYDRATE CONTENT OF TRICHODERMA CELLULASES

ENZYME[a]	MOL. WT. (Daltons)	ORGANISM[b]	CARBOHYDRATE CONTENT (WT%)	SUGARS[#] (No./Molecule)	REF.
CELLOBIOHYDROLASES					
CBH [pI 3.80]	--	T. koningii	9	N.D.	50
CBH [pI 3.95]	--	T. koningii	33	N.D.	50
CBH IV	46000	T. viride	3.3	N.D.	51
CBH	42000	T. viride	9.2	Man(16)* Glc(6)	52
CBH A [I(A)]	53000	T. viride	1.4	Man(3)* Glc(1)	48
CBH B [I(B)]	53000	T. viride	5.8	Man(12)* Glc(5)	48
CBH C [I(C)]	53000	T. viride	10.4	Man(22)* Glc(6) Gal(3)	48
CBH D [I(D)]	53000	T. viride	6.7	Man(13)* Glc(7)	48
CBH C [I(C)]	48400	T. viride		Man(26.4) Glc(4.8) Gal(2.4) $GlcNH_3$(3.4)	30
CBH I(D)	54000	T. reesei QM 9414	4.3	Man(13) Glc(4) GlcNac(4)	49
CBH II	54000	T. reesei QM 9414	19.5	Man(51) Glc(20)	49

ENDOGLUCANASES

ENDO 1	13000	T. koningii	N.D.	N.D.	53
ENDO 3a	48000	T. koningii	N.D.	N.D.	53
ENDO 3b	48000	T. koningii	N.D.	N.D.	53
ENDO 4	31000	T. koningii	N.D.	N.D.	53
ENDO	51000	T. viride QM 9414	0	--	57
ENDO	20000	T. viride QM 9414	0	--	58
ENDO I	12500	T. viride	21	N.D.	54
ENDO II	50000	T. viride	12	N.D.	54
ENDO II	37200	T. viride	4.5	Man(5)* Gal(2) Glc(2)	55
ENDO III	52000	T. viride	15	Man(33)* Gal(5) Glc(6)	55
ENDO IV	49500	T. viride	15.2	Man(30)* Gal(6) Glc(6)	55
ENDO	46700	T. reesei QM 9414	10.6	Man(32) Glc(9)	49

a) Enzymes are named according to the original publication referenced; some enzymes with identical primary structure may have been given different names.
b) T. viride QM 9414 and T. reesei QM 9414 are the same organism [59].
#) Number of residues per protein molecule.
*) Number of residues calculated from weight percent data in original reference.

glycoenzyme. All the neutral carbohydrate was found to be released from cellobiohydrolase C from Trichoderma reesei QM 9414, with alkaline borohydride (reductive ß-elimination), whereas the amino sugars remained attached to the polypeptide. It was calculated that the neutral carbohydrate was attached to serinyl and threoninyl residues at an average of 16.7 sites per enzyme molecule. A range of small oligosaccharides were released, with the mono- and trisaccharides being the most abundant. Periodate oxidation and G.C./M.S. of partially methylated residues determined that (i) the oligosaccharides were unbranched, (ii) most of the linkages between the neutral sugars in the chains are (1-6) and (iii) mannose was the sugar linking the oligosaccharides to the hydroxy amino acids. Glycosidase experiments suggested that α-mannose was present at the non-reducing ends of some chains and that the disaccharides were mannobiose.

More recently, Fägerstam et al. [60] have sequenced most of CBH I(D) and found that all of the O-linked carbohydrate was attached within a short region, 20 amino acids in length, located 32 residues from the C-terminus of the polypeptide. Due to the presence of the carbohydrate, this short region was never sequenced, but was found to contain seven threonyl and three seryl residues. Shoemaker et al. [61] have now sequenced the gene encoding CBH I obtained from Trichoderma reesei strain L27. The nucleic acid sequence was shown to contain two introns and, by comparison to the previously available amino acid sequence, the region of carbohydrate attachment was shown to contain eight threonyl and three seryl residues.

The ß-glucosidases produced by Trichoderma have been found to contain little or no carbohydrate. Berghem and Pettersson [62] reported no carbohydrate attached to an extracellular ß-glucosidase from T. reesei using an orcinol-sulfuric acid method. Chirico [44] found that a ß-glucosidase from the same organism would not bind to Concanavalin A-Sepharose, indicating the lack of available α-glucosyl or α-mannosyl residues. However, four residues of neutral sugar and two residues of N-acetylglucosamine were estimated per enzyme molecule based on phenol-sulfuric acid and amino sugar analysis, respectively. The identity of the neutral sugar was not determined. This was quite different from the enzyme isolated by Emert [63] from T. viride, which was found to contain 10.3% carbohydrate. Gong et al. [56] also determined that a cellobiase (ß-glucosidase) activity from another T. viride preparation did not bind to Concanavalin A-Sepharose. Wood and McCrae [64] purified two ß-glucosidases, BG1 and BG2, from Trichoderma koningii. Neither enzyme bound to Concanavalin A-Sepharose although BG2 was shown to contain 2% neutral carbohydrate by a phenol-sulfuric acid determination. By the same method, no carbohydrate was found associated with BG1.

Glycosylated cellulases are also produced by organisms other than Trichoderma, although no detailed carbohydrate analyses have been performed on them. Eriksson and Pettersson [65] have purified five endoglucanases from the white-rot fungus, Sporotrichum pulverulentum, of which four are glycosylated. The four glycoenzymes, T_1, T_{2b}, T_{3a} and T_{3b} were found to contain 10.5%, 7.8%, 4.7% and 2.2% carbohydrate, respectively, as determined by gas-liquid chromatography as the alditol acetates. T_1 was found to have 19 mannosyl and

2 glucosyl residues and T_{2b} was found to have 5 mannosyl, 7 galactosyl, 1 glucosyl and 1 arabinosyl residue per enzyme molecule. Kanda et al., using the phenol-sulfuric acid method, showed that both an endocellulase [66] and an exocellulase [67] from the fungus Irpex lacteus were glycosylated, containing 12.2% and 2.4% carbohydrate, respectively. Beguin and Eisen [68] purified three endocellulases, I, CA and CB, from Cellulomonas. While enzymes CA and CB were found to stain with periodic acid-fuchsin and also bound to Concanavalin A-Sepharose, typical of glycoproteins, enzyme I did neither and was proposed to be unglycosylated. The amount and nature of the carbohydrate were not determined. Tong et al. [69] determined that three cellulases, I, II and III, and a ß-glucosidase from the thermophilic fungus Thermoascus aurantiacus, were all glycosylated based on the results of anthrone-sulfuric acid analysis. They were found to contain 33%, 5.5%, 2.6% and 1.8% carbohydrate, respectively. Ait et al. [70] found that electrophoretic gels of a cellulase from Clostridium thermocellum stained with periodic acid-fuchsin suggesting that it also is glycosylated.

Wood and McCrae [71] separated four cellobiohydrolase forms, A, B, C and D, from Fusarium solani, by isoelectric focussing. Phenol-sulfuric acid analysis indicated that they contained 21%, 10%, 12% and 1% carbohydrate, respectively. These same workers later compared the carbohydrate composition of this cellobiohydrolase activity with that of cellobiohydrolases from Penicillium funiculosum and T. koningii [72]. Of the carbohydrate attached to F. solani cellobiohydrolase (all four species), 83% was mannose, 7% was xylose, 4% was glucose and 1% was galactose. The carbohydrate component of the

cellobiohydrolase activity of T. koningii (two species) was found to be composed of 73% mannose, 27% glucose and a trace amount of xylose. The structure of the carbohydrate of any of these enzymes has not been reported.

Whereas the ß-glucosidases of Trichoderma are either not glycosylated or are so at a low level [44,56,62-64], enzymes from other species are reported to be glycosylated to a greater extent. Shewale and Sadana [73] purified four ß-glucosidases from the fungus Sclerotium rolfsii and suggested that they were glycoproteins due to their affinity for Concanavalin A-Sepharose and to a positive reaction to periodic acid-fuchsin staining. Rudick and Elbein [74] determined that a ß-glucosidase from Aspergillus fumigatus contained glucosamine and mannose based on the results of paper and gas-liquid chromatography. Approximately 15 moles of mannose and 2 moles of glucosamine were found per mole protein. That either sugar could only be released by strong alkaline treatment with 1N NaOH at 100^{o}C for 6 hours suggested that the carbohydrate was associated with the protein via a glucosaminyl-peptide linkage. Hirayama et al. [75] purified a glycoprotein ß-glucosidase from the phytopathogenic fungus, Pyricularia oryzae. Although the enzyme contained 101 mannosyl and 13 glucosyl residues per protein molecule, as determined by gas-liquid chromatography of the trifluoroacetyl derivatives, only 5% of the carbohydrate could be selectively removed using an α-mannosidase.

Structure:Function Relationships for Fungal Glycoenzymes

While little is known about the structure of the carbohydrate attached to fungal cellulases, even less is known about its function. Hayashida and Yoshioka [76] were able to partially remove the carbohydrate attached to an exocellulase (Avicelase) and an endocellulase (CMCase) from the thermophilic fungus Humicola insolens YH-8 by either chemical or enzymatic treatment. Treatment of either with a mixed-glycosidase preparation was shown to release about 65% of the carbohydrate. The removal of the carbohydrate by this method did not appear to affect the ability of either enzyme to hydrolyze crystalline cellulose (Avicel), although the thermal and pH stability of both were decreased. Treatment with periodate oxidation and Smith degradation was shown to release about 90% of the carbohydrate from each enzyme. After this chemical degradation, activity of each on Avicel was decreased about 30% and pH and thermal stability of each was also further reduced. There was no apparent effect of covalently bound carbohydrate on substrate specificity since cellobiose was the predominant product of enzymatic hydrolysis of cellulose by either the native, 65%-carbohydrate depleted or 90%-carbohydrate depleted exo- or endocellulase. However, in neither treatment was all the carbohydrate removed and in the case of the chemical treatment, the effect on the polypeptide was not determined. These results suggest that the carbohydrate is important primarily for stability of the enzymes.

Extensive periodate oxidation of glucoamylase I from Aspergillus niger was shown to cause a marked loss in the stability of the enzyme [77]. Glucoamylase I, normally heavily glycosylated at

about 45 sites on the polypeptide, was demonstrated to precipitate out of solution when two-thirds of the carbohydrate was removed by this method. Thus, for this enzyme, solubility and perhaps transfer to the aqueous phase of the culture medium may be affected significantly by the glycosylation.

To understand the importance of carbohydrate covalently-bound to glycoproteins it is necessary to know the role of the protein to which it is attached. Whereas the role of some glycoproteins is not known, glycoenzymes offer a dynamic function for which one can study binding and catalysis. Thus, definitive information can be achieved either by preventing glycosylation or by removing the carbohydrate from the post-translationally modified polypeptide. In spite of our knowledge concerning the enzymic properties of fungal glycoenzymes, and cellulases in particular, there is little known regarding the three-dimensional structure of these enzymes. If the carbohydrate can be removed from these glycoenzymes, the polypeptide may be more amenable to crystallization and X-ray diffraction studies.

Assessment

Physico-chemical studies have revealed the molecular weight, isoelectric point, amino acid composition and more recently, the amino acid sequence of CBH I(D) and the genetic sequence encoding the information for synthesis of CBH I(D) messenger RNA. Peptide isolation has shown that the O-glycosylated residues are confined to a short sequence on the protein. With the availability of sophisticated instrumentation such as high resolution nuclear magnetic resonance

(NMR) and gas chromatography/mass spectrometry (GC/MS), the stucture of oligosaccharides attached to glycoproteins can be accurately elucidated. The role the carbohydrate plays in cellulase function cannot be fully understood unless the structure is known; this information will enable further exploration of the molecular basis for cellulase biosynthesis, secretion, stability, activity and microheterogeneity. Successful analyses, of course, depend on pure preparations of proteins and oligosaccharides to ensure a definitive structural description.

EXPERIMENTAL PROCEDURES

Materials

Enzymes--Crude extracellular preparation from Trichoderma reesei QM 9414, grown on Avicel (microcrystalline cellulose), was a gift from Gulf Oil Chemicals Company, Merriam, KS.

ß-Glucosidase from Trichoderma reesei QM 9414; prepared by William Chirico.

α-Glucosidase (maltase) from Yeast (Lot #202900); Calbiochem, Los Angeles, CA.

α-Glucosidase (Type I) from Yeast (Lot #39B-5370), α-glucosidase (Type VI) from Yeast (Lot #21F-8106), pullulanase from Enterobacter aerogenes (Lot #99C-02731), amyloglucosidase from Aspergillus niger (Lot #72F-0560); Sigma Chemical Company, St. Louis, MO.

α-Mannosidase from Jack Bean (Lot #7064101); Boehringer Mannheim Corporation, New York, NY.

Chromatographic Supplies--DEAE-Sephadex A-50, SP-Sephadex C-50; Pharmacia Fine Chemicals, Piscataway, NJ.

Dowex 50W (200-400 mesh; H^+ form), Biogel P-2 (-400 mesh); BioRad Laboratories, Richmond, CA.

Amberlite MB-3 (mixed bed resin, Amberlite IR-120 and Amberlite IRA-410, fully regenerated); Mallinckrodt, Inc., Paris, KY.

OV-225 (cyanopropylmethyl phenyl-methyl silicone), Chromosorb G-HP (80-100 mesh); Varian Associates, Sunnyvale, CA.

Neopentyl glycol succinate; Alltech Associates, Deerfield, IL.

Gas Chrom Q (100-120 mesh); Applied Science, State College, PA.

Whatman Partisil PXS 10/25 PAC column (bonded cyano-amino type, polar phase); Whatman Chemical Separations Division, Clifton, NJ.

Substrates--CM-Cellulose 7HP; Hercules Powder Company, Wilmington, DE.

Avicel PH 101 (microcrystalline cellulose); American Viscose Division, FMC Corporation, Newark, DE.

Walseth cellulose (phosphoric acid-swollen cellulose); prepared from Avicel PH 101 by the method of Wood [78].

p-Nitrophenyl-α-D-mannopyranoside (Lot #701993); Calbiochem, Los Angeles, CA.

p-Nitrophenyl-α-D-glucopyranoside (Lot #09053-3); Pierce Chemical Company, Rockford, IL.

p-Nitrophenyl-β-D-mannopyranoside (Lot #62C-1270), p-nitrophenyl-β-D-glucopyranoside (Lot #88C-5039); Sigma Chemical Company, St. Louis, MO.

Carbohydrate Standards--Dextrose; National Bureau of Standards, Washington, DC.

D-Xylose, D-xylitol, D-galactose, gentiobiose, Yeast mannan; Sigma Chemical Company, St. Louis, MO.

D-Mannose; Calbiochem, Los Angeles, CA.

Cellobiose; Eastman Kodak Company, Rochester, NY.

Kojibiose, nigerose; gifts from Dr. Seiya Chiba, Department of Agricultural Chemistry, Hokkaido University, Sapporo, Japan.

Isomaltose; Applied Science Laboratories, Inc., State College, PA.

Dextran T-10; Pharmacia Fine Chemicals, Piscataway, NJ.

Chemicals--Acetic Anhydride; Mallinckrodt, Inc., Paris, KY.

Acetonitrile (HPLC grade), dimethyl sulfoxide (kept dry over 3A molecular seives, methyl iodide, palladium (II) Chloride, hydroxylamine hydrochloride; Fisher Scientific Company, Fair Lawn, NJ.

Acrylamide (>99.9%); BioRad Laboratories, Richmond, CA.

Ampholytes (Ampholine,pH 3.5-9.5); LKB Produkter AB, Bromma, Sweden.

Basic Fuchsin (91% dye); Eastman Kodak Company, Rochester, NY.

Bromophenol Blue; Canalco, Rockville, MD.

Coomassie Brilliant Blue R250, Column Coat, N,N-methylene-bis-acrylamide, ammonium persulfate; Miles Laboratories, Inc., Elkhart, IN.

Deuterium oxide (99.96 atom % D); Aldrich Chemical Company, Inc., Milwaukee, WI.

Disodium succinate hexahydrate (A grade); Calbiochem, Los Angeles, CA.

Periodic acid; G. Frederick Smith Chemical Company, Columbus, OH.

Pyridine (silylation grade), N,N,N',N'-tetramethylethylene diamine (TEMED); Pierce Chemical Company, Rockford, IL.

Riboflavin, L-α-amino-n-butyric acid, Freunds Complete Adjuvent; Sigma Chemical Company, St. Louis, MO.

Sodium borohydride, sodium hydride (50% in oil dispersion); Alfa Products, Danvers, MA.

All other chemicals were reagent grade.

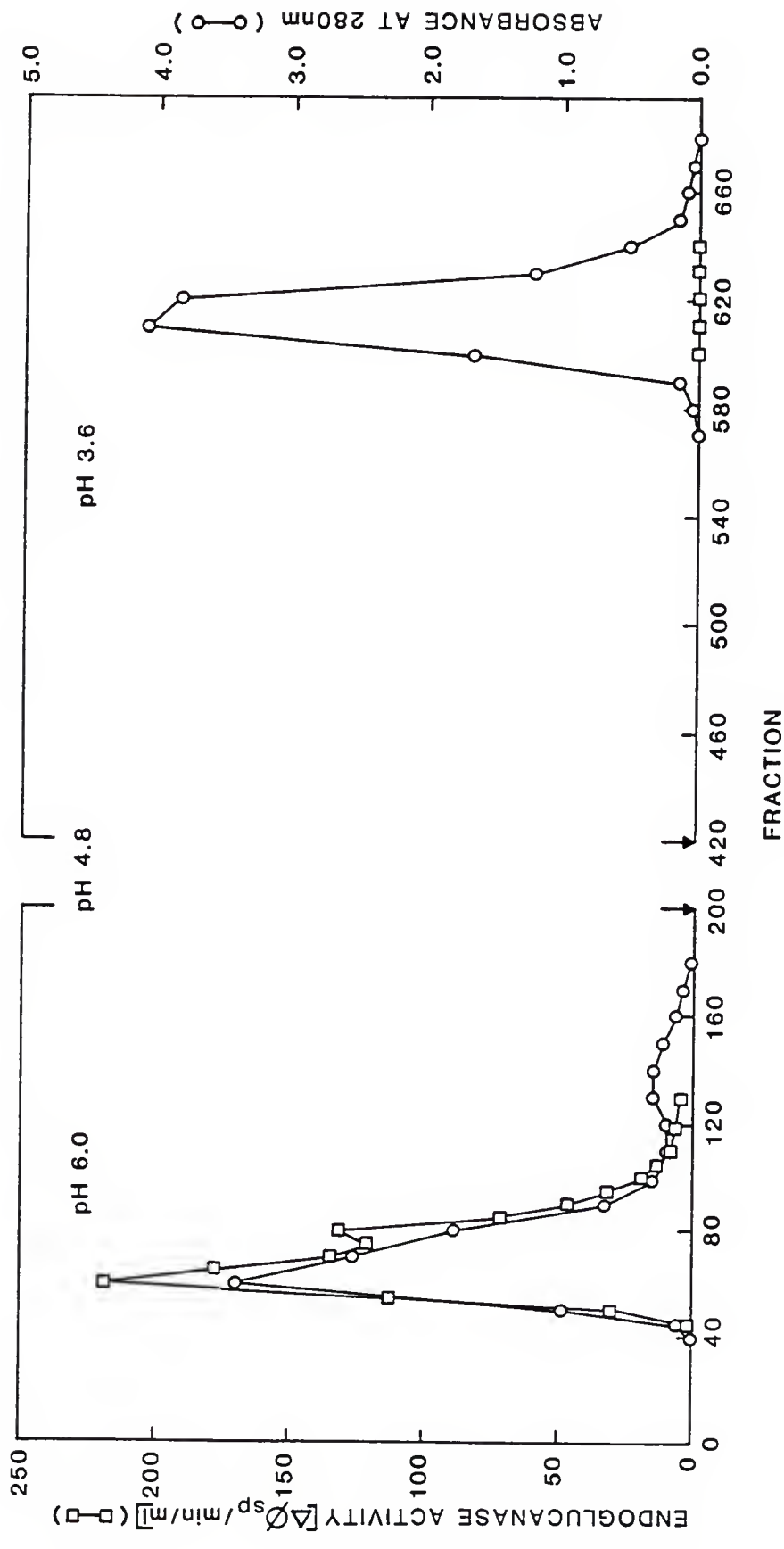
Methods

Purification of Cellobiohydrolase I(D)--Cellobiohydrolase I(D) (CBH I(D)) was purified from an extracellular culture filtrate of Trichoderma by a modification of the method described by Gritzali [49]. A crude precipitate produced by adding ammonium sulfate (65% saturation) to a solution of the extracellular material from Trichoderma reesei QM 9414 grown on Avicel was used as the source of the enzyme. This precipitate was dissolved in distilled water, dialyzed and lyophillized. The resulting pale yellow powder (5.0-7.5 g) was dissolved in 150 ml of 50 mM sodium succinate, 3 mM sodium azide, pH 6.0. The mixture was then filtered slowly through glass fibre paper (Whatman, grade 934 AH) and the filtrate applied to a DEAE-Sephadex A-50 column (14 x 22 cm) equilibrated with the same buffer. Cellobiohydrolase II, aryl-ß-D-glucosidase and endo-1,4-ß-D-glucanase activity were eluted isocratically at a flow rate of 400 ml/h (Fig. 1). CBH I(D) was obtained in high purity when the pH of the elution buffer was lowered in a stepwise fashion to 3.6. The previous method [49] involved elution of CBH I(D) in the presence

FIGURE 1

Isocratic elution pattern from DEAE-Sephadex column chromatography of the extracellular protein preparation from T. reesei QM 9414 grown on cellulose.

Conditions of elution were described in Experimental Procedures. The volume of each fraction was 25 ml.


ENDOGLUCANASE ACTIVITY [ΔØsp/min/ml] (□—□)
ABSORBANCE AT 280nm (○—○)
FRACTION
pH 6.0
pH 4.8
pH 3.6
0
40
80
120
160
200
420
460
500
540
580
620
660
50
100
150
200
250
1.0
2.0
3.0
4.0
5.0
0.0

of 0.5M NaCl, and while this was adequate for that cellulase preparation, with this batch under the same conditions, several impurities eluted with the enzyme. In the absence of the salt, CBH I(D) eluted at pH 3.6 with approximately one bed volume of buffer and was separated from minor contaminants.

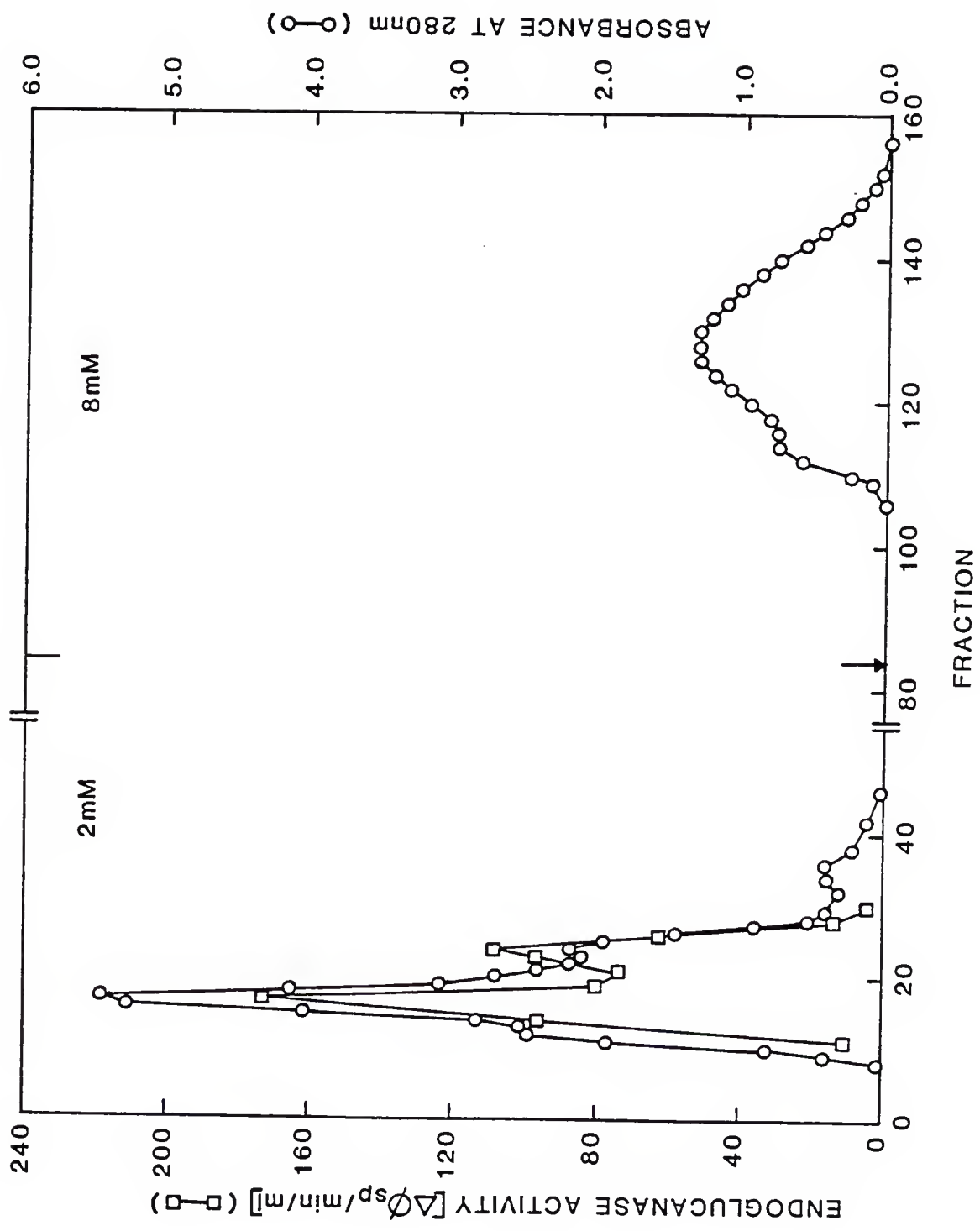
Purification of Cellobiohydrolase II--Cellobiohydrolase II (CBH II) was also purified by the method of Gritzali [49]. The proteins eluting isocratically at pH 6.0 from the DEAE-Sephadex column used to separate CBH I(D) (fractions 45-105) were pooled. This material, containing CBH II, endoglucanase and ß-glucosidase activity, was dialyzed against 2mM sodium succinate containing 3mM sodium azide at pH 4.5, concentrated by ultrafiltration to 30 ml and applied to an SP-Sephadex C-50 column (4.4 x 55 cm). All endoglucanase activity eluted isocratically (Fig. 2) and CBH II was obtained in high purity when eluted in a batchwise manner with an elutant of 8mM sodium succinate containing 3mM sodium azide at pH 4.5 (flow rate = 70 ml/h). The previous method [49] eluted these enzymes in the same buffer at pH 5.0, but elution at the lower pH was conducted in this case to better separate the endoglucanases for further purification. ß-Glucosidase activity was subsequently eluted from the column with 50mM sodium succinate, 3mM sodium azide, pH 6.0.

Dialysis and Ultrafiltration--Protein concentration and dialysis were performed on either an Amicon Model 2000 High Performance Ultrafiltration Cell (volumes up to 2 l) or an Amicon Model 202 Ultrafilration Cell (volumes up to 200 ml)(Amicon Scientific

FIGURE 2

Isocratic elution pattern from SP-Sephadex column chromatography of cellobiohydrolase II and the endoglucanases which had been eluted from a DEAE-Sephadex column.

Conditions of elution are described in "Experimental Procedures." The volume of each fraction was 21.0 ml.


ENDOGLUCANASE ACTIVITY [ΔØsp/min/ml] (□—□)
ABSORBANCE AT 280nm (○—○)
FRACTION
2mM
8mM
0
40
80
120
160
200
240
0.0
1.0
2.0
3.0
4.0
5.0
6.0
20
40
80
100
120
140
160

Systems, Lexington, MA). Pressure was supplied with nitrogen gas at 40 psi and 30 psi, respectively. A Diaflo PM-10 Ultrafilter, with a molecular exclusion limit of 10,000 daltons, was used with each cell.

Protein Determination--Protein concentrations were estimated during purification by spectrophotometric determination at 280 nm, using an approximate extinction coefficient ($E^{1\%}$ at 280 nm) of 10.0. For all quantitative analyses, protein concentration was determined using experimentally derived extinction coefficients. Samples of protein were dialyzed extensively against water, lyophillized and then stored over phosphorous pentoxide for at least 10 days. Protein was weighed and then made up into solutions of known concentrations of between 0.3-0.8 mg/ml. The absorbance of each solution was determined both at 260 nm and 280 nm on a Beckman DU-8 UV-Visible Spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA).

Enzyme Assays--Endo-1,4-ß-D-glucanase activity was measured by following the decrease in viscosity of a carboxymethylcellulose solution as described by Shoemaker and Brown [79]. Specific activity was expressed as the change in specific fluidity/min/mg protein. Aryl-glycosidase activity was followed by monitoring the release of p-nitrophenol from p-nitrophenyl-glycosides. A solution of 10 mM substrate (0.5 ml) and an appropriate buffer (2.0 ml) were preincubated in small test tubes for 10 min at 40°C. For α-mannosidase, digestions were performed with 50mM sodium citrate containing 3mM sodium azide at pH 4.5 and for α-glucosidase reactions were carried out in solutions of 65mM potassium dihydrogen phosphate

with 3mM sodium azide at pH 6.0. Aliquots of enzyme solution (2-25 µl) were then added, mixed and incubated at 40°C for 20 min. The tubes were placed in a boiling water bath for 10 min, and when cool, 1 ml sodium borate (0.5 M, pH 9.8) was added. The absorbance at 400 nm was measured, compared to a p-nitrophenol standard curve and specific activity expressed as µmoles p-nitrophenol released/min/mg protein. The solution was adjusted with the sodium borate buffer to pH 9.1, a value greater than one pH unit above the pKa of p-nitrophenol, assuring complete dissociation.

Polyacrylamide Disc Gel Electrophoresis--Disc gel electrophoresis was performed as a routine analysis of column fractions during purification and was used as a criterion of homogeneity. Electrophoresis was conducted using the discontinuous buffer system No. 1 described by Maurer [80]. The pH of the stacking gel and separating gels were 8.3 and 9.5, respectively, and the polyacrylamide concentration was 7.5 percent (W/V). Power was supplied at 2 mA/tube until the bromophenol blue tracking dye had entered the separating gel, at which point it was increased to 3 mA/tube until the dye band was within 0.5 cm of the bottom of the tube. Separations were performed in a Canalco electrophoresis chamber (Miles Laboratories, Inc., Elkhart, IN) with a Hoefer PS 1200 DC power supply (Hoefer Scientific Instruments, San Fransisco, CA).

After electrophoresis, protein bands were fixed by immersion in 12 percent (W/V) trichloroacetic acid for 30 min after which the gels were washed with water (3 x 5 min). Protein was stained with 0.1% Coomassie Brilliant Blue R250 in methanol-water-acetic acid (45:45:10)

for 1 h and subsequently destained with 7% acetic acid at 40°C with frequent changes of destaining solution. Carbohydrates in the gels were stained by the periodic acid-Schiff (PAS) method described by Lang [81]. After fixing and washing, the gels were immersed in fresh periodic acid (0.5% w/v) for 1 h in the dark. The acid was removed by successive washes with 7% acetic acid (3 x 10 min). Gels were then stained for 1 h with 1% Basic Fuchsin in 0.15N HCl containing 1.9% sodium metabisulfite. Excess dye was removed by destaining with 0.1% sodium metabisulfite at 40°C, with repeated changes of destaining solution.

Isoelectric Focusing--Isoelectric points for homogeneous proteins were determined using a BioRad Model 1415 Horizontal Electrophoresis Cell (BioRad Laboratories, Richmond, CA). Determinations were performed with cold water (4°C) passing through the gel bed using a Haake Model FE Constant Temperature Circulator (Haake Instruments, Inc., Saddle Brook, N.J.). Polyacrylamide gel slabs (100 x 125 x 0.8 mm) were used as described by BioRad [82], without initial prefocusing and with the modification that the ampholytes were replaced with 0.5 ml Ampholine, pH 3.5-9.5 (LKB Produkter AB, Bromma, Sweden), as these produced more linear pH gradients. Electrofocusing was performed for 2.5 h at 4.5 amps constant current. Best results were obtained using 10µg samples of protein applied to small strips of glass fiber paper (0.3 x 0.5 cm) placed at the center of each lane on the gel slab. The strips were removed 30 min after the current was turned on. After electrofocusing, sections were cut (0.5 x 0.5 cm) down each side of the gel and placed

in tubes containing 1 ml 0.1M KCl (made with degassed, deionized water). The pH of each section was determined after 1 hour at room temperature and the gradient calculated. Fixing, staining and destaining were performed as described by Winter et al. [83]. Proteins were fixed (17.3 g sulfosalicylic acid and 57.5 g trichloroacetic acid in 500 ml water) for 1 h at room temperature, stained with Coomassie Brilliant Blue R250 (0.46 g in 400 ml destaining solution) for 1 h and then destained (water-ethanol-acetic acid, 67:25:8) until the background was clear.

Carbohydrate Composition--Total neutral carbohydrate was determined by the phenol-sulfuric acid method of Dubois et al. [84]. Carbohydrate components of the enzymes were determined after either hydrolysis or ß-elimination and individual monosaccharides were identified after separation by gas-liquid chromatography. Aldoses were determined as the peracetylated aldononitriles (PAANs) and alditols were determined as the peracetylated alditols (PAAs).

For phenol-sulfuric acid analysis, 0.1-1.0 mg of glycoprotein (containing 2-15 µg neutral carbohydrate) was dissolved in 0.2 ml water in a small test tube. Phenol (0.2 ml,3%) was added followed by 1.0 ml concentrated H_2SO_4 with vigorous mixing using a vortex mixer. The samples were cooled to 40°C and the absorbance determined at 400 nm, using mannose as the standard, as this is the principal carbohydrate constituent of these glycoproteins.

Lyophillized glycoproteins (1-2 mg) were hydrolyzed in 1 N HCl (2 ml) for 2 h at 100°C in sealed 5 ml Reacti-Vials (Pierce Chemical Company, Rockford, IL). When cool, xylose and/or xylitol were added

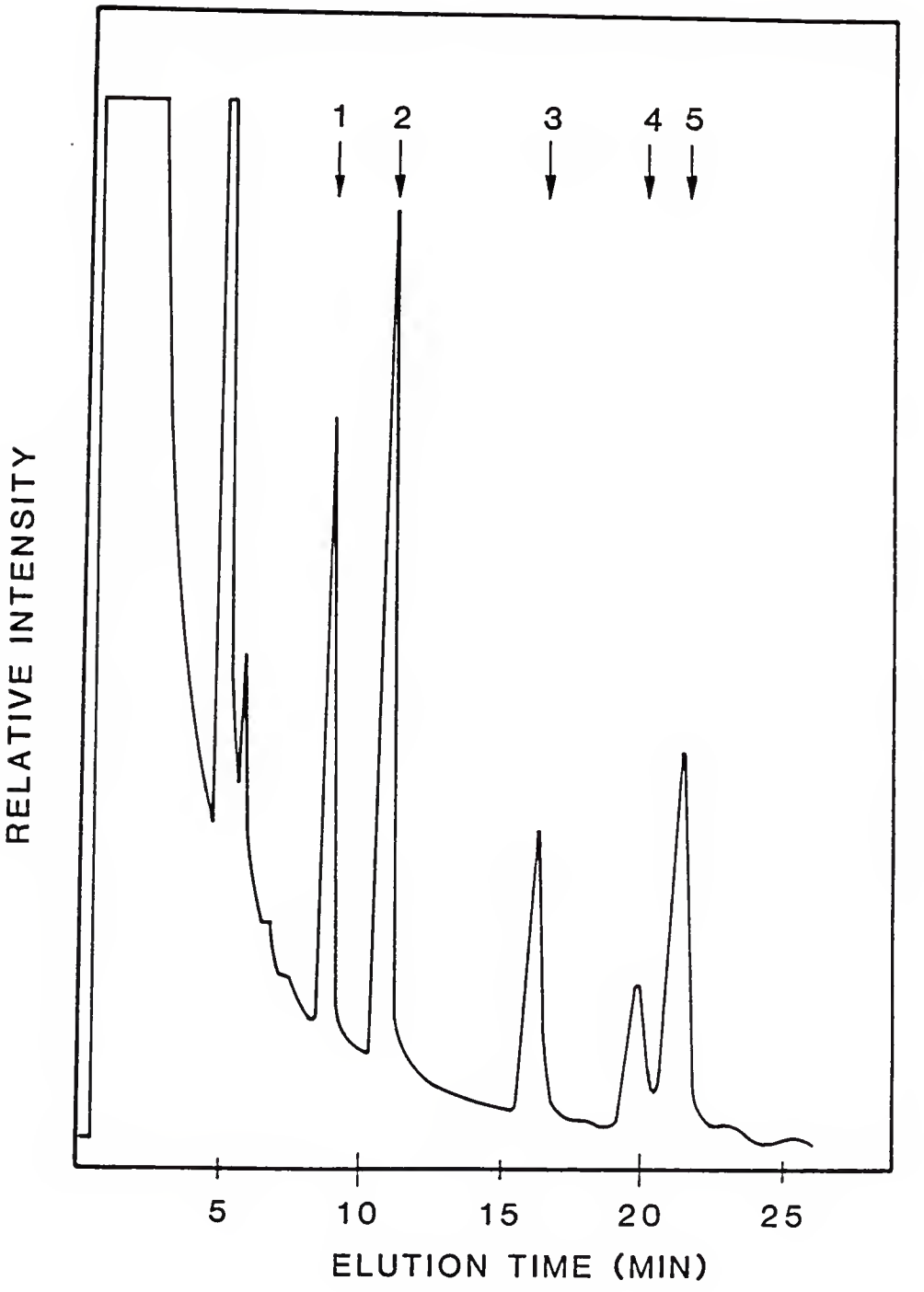
as internal standards and the mixtures immediately deionized using small columns (1.4 x 6 cm) of Amberlite MB-3 mixed bed resin. The eluants were taken to dryness in a rotary evaporator, transferred to 5 ml Reacti-Vials and lyophillized. Monosaccharides were then either converted to the peracetylated aldononitriles (PAAN) by a modification of the method of Varma et al. [85] or subsequently reduced and converted to the peracetylated alditol by the method of Sawardeker et al. [86]. It was essential that these derivatives be separable and a profile of that separation by gas-liquid chromatography is shown (see Fig. 3).

The PAAN preparative procedure involved adding 3-5 mg hydroxylamine hydrochloride and 0.25 ml pyridine to the lyophillized monosaccharide mixture (0.01-10mg), sealing the Reacti-Vials with screw top teflon caps and heating at 90°C for 45 min. The resultant oximes were then peracetylated by addition of 0.25 ml acetic anhydride and heating at 90°C for 45 min. The products were taken to dryness under nitrogen at 60°C and then evaporated with toluene (3 x 1 ml) to remove residual acetic anhydride using an N-Evap Model 106 Analytical Evaporator (Organomation Associates, Inc., Shrewsbury, MA). The products were subsequently partitioned between 3M HCl (1 ml) and $CHCl_3$(1 ml), the aqueous layer removed and the organic layer washed with water (2 x 1 ml). Washing involved vigorously mixing the two phases in the Reacti-Vial and removing the aqueous layer with a pipette after partitioning was complete. The $CHCl_3$ layers were then washed with 0.5M sodium bicarbonate (1 x 1 ml) and then again with water (1 x 1 ml). A spatula tip of $MgSO_4$ powder was then added to each to remove any excess water. The organic layers were then filtered

FIGURE 3

Gas chromatographic separation of peracetylated alditol and aldononitrile derivatives of neutral monosaccharides released from cellobiohydrolase I(D) after reductive ß-elimination and subsequent acid hydrolysis.

Peaks identified are peracetylated 1. xylononitrile; 2. xylitol; 3. mannononitrile; 4. gluconononitrile; 5. mannitol. Conditions of separation are described in "Experimental Procedures". The peaks eluting before the xylononitrile acetate peak were always present in PAAN preparations but not in PAA preparations and are thought to be byproducts of the reaction [88].


1
2
3
4
5
RELATIVE INTENSITY
5
10
15
20
25
ELUTION TIME (MIN)

through glass wool, concentrated to about 20-50 µl and 1 µl aliquots were analyzed by gas-liquid chromatography.

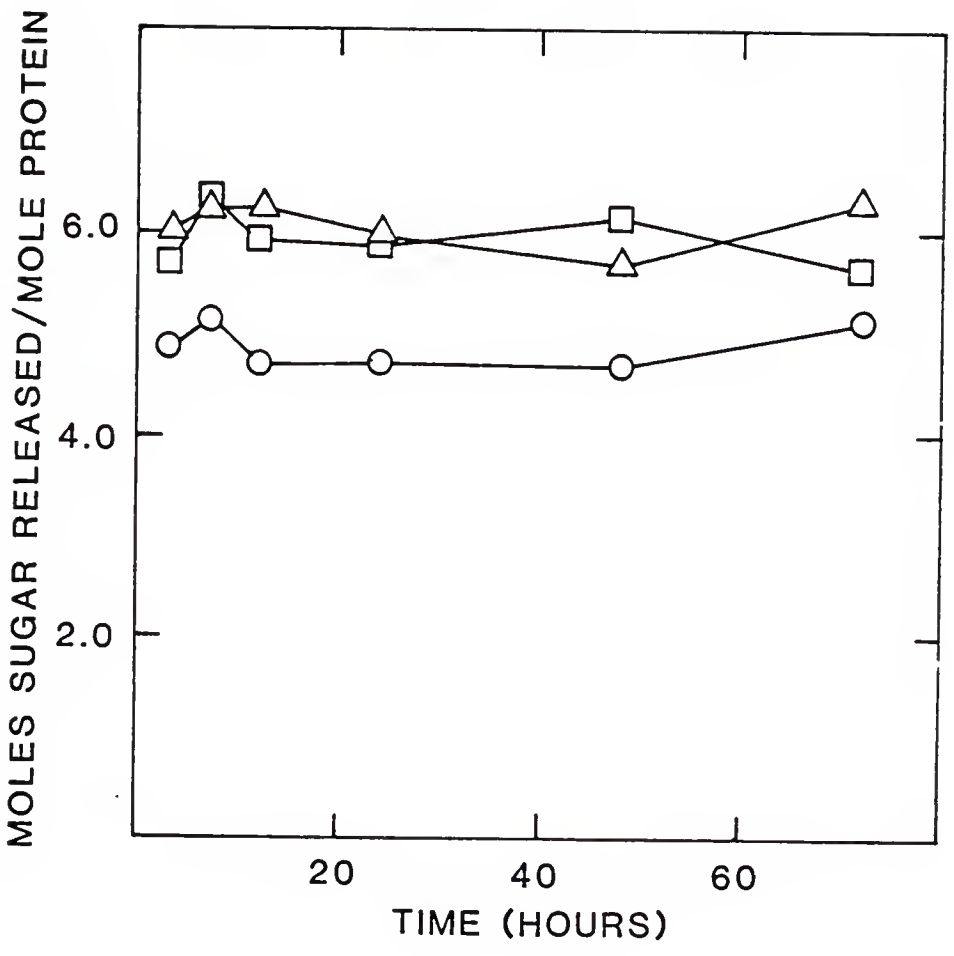
In the procedure for peracetylated alditols, monosaccharides were reduced overnight with aqueous sodium borohydride (10 mg in 1 ml water) at 4°C. The reactions were stopped by dropwise addition of 2N acetic acid until the effervescence stopped, using 3 drops of n-octanol to suppress foaming. The mixtures were taken to dryness under a stream of dry nitrogen at 60°C and the residues evaporated with methanol (3 x 1 ml) to remove excess borate as the volatile methyl ester. The mixtures were then deionized by passage through small Amberlite MB-3 columns and the eluants were taken to dryness on a rotary evaporator. The samples were transferred to 5 ml Reacti-Vials and lyophillized. Pyridine (0.25 ml) and acetic anhydride (0.25 ml) were added and the vials sealed and heated at 90°C for 45 min. The mixtures were taken to dryness under a stream of dry nitrogen at 60°C and then evaporated with toluene (3 x 1 ml). The residues were dissolved in 20-50 µl $CHCl_3$ and 1 µl aliquots analyzed by gas-liquid chromatography.

O-linked oligosaccharides were released by reductive ß-elimination of the glycoproteins, by a modification of the method of Nakajima and Ballou [87]. To 1 or 2 milligrams of the glycoproteins in a 5 ml Reacti-Vial were added 2 ml of 0.1N NaOH and 0.3M $NaBH_4$. The solution was incubated at 40°C for 48 hours. During a 72 h control experiment, no further sugars were released from the protein after 3 hours of reductive ß-elimination (Fig. 4). After neutralization by dropwise addition of 2 N acetic acid, the samples were evaporated with methanol to remove borate esters and then

FIGURE 4

Efficacy of sugar release from cellobiohydrolase I(D) by reductive ß-elimination.

Oligosaccharides released were subsequently hydrolyzed to constituent sugars. The resultant monosaccharides were then converted to the peracetylated alditols and aldononitriles and analyzed by gas-liquid chromatography on 1% OV-225 as described under "Experimental Procedures". △, Mannose; □, Mannitol; ○, Glucose.


MOLES SUGAR RELEASED/MOLE PROTEIN
6.0
4.0
2.0
20
40
60
TIME (HOURS)

deionized by passage over Amberlite MB-3. The eluate was dried under reduced pressure and hydrolyzed as previously described. The sugars released were converted to either a mixture of PAANs and PAAs by the PAAN method described previously or were further reduced with $NaBH_4$ and all converted to the PAAs. Formation of mixed derivatives is feasible as alditols, unlike aldoses, do not form the corresponding nitrile in the presence of pyridine and hydroxylamine hydrochloride; but do undergo peracetylation upon addition of acetic anhydride. This derivatization method following ß-elimination provided information regarding, not only the total neutral carbohydrate composition of the O-linked oligosaccharides, but also identification of the type of sugar attached to the protein (leading to the formation of the corresponding PAA) and an estimation of the number of sites at which carbohydrate had been attached to the polypeptide.

The area of peaks resulting from gas-liquid chromatography (see below) of the sugar derivatives were subjected to integration and were compared to internal standards. Positive identification of derivatives was achieved by gas chromatography/mass spectrometry (see below). Quantitative determination of the peracetylated aldononitriles of mannose and glucose were derived from the linear relationship of their GLC peak areas with respect to that of the xylose internal standard as were those of the peracetylated alditols of mannitol and glucitol with respect to the peracetylated xylitol internal standard (Figs. 5 and 6).

While alditol acetate formation proceeds essentially to completion, Furneaux [88] has shown that formation of some peracetylated aldononitriles occurs with the formation of several

FIGURE 5

Quantitation of aldoses as the peracetylated aldononitriles.

Aldoses (mannose (○), glucose (●) and xylose (internal standard)) were converted to the PAANs and the derivatives were separated by gas-liquid chromatography on 1% OV-225 at 190°C as described under "Experimental Procedures". The ratio of peak areas of sample/standard was plotted against the molar ratio of the sample/standard. Correction factors necessary for calculation of unknowns were determined to be: Man/Xyl, 0.85; Glc/Xyl, 0.94.

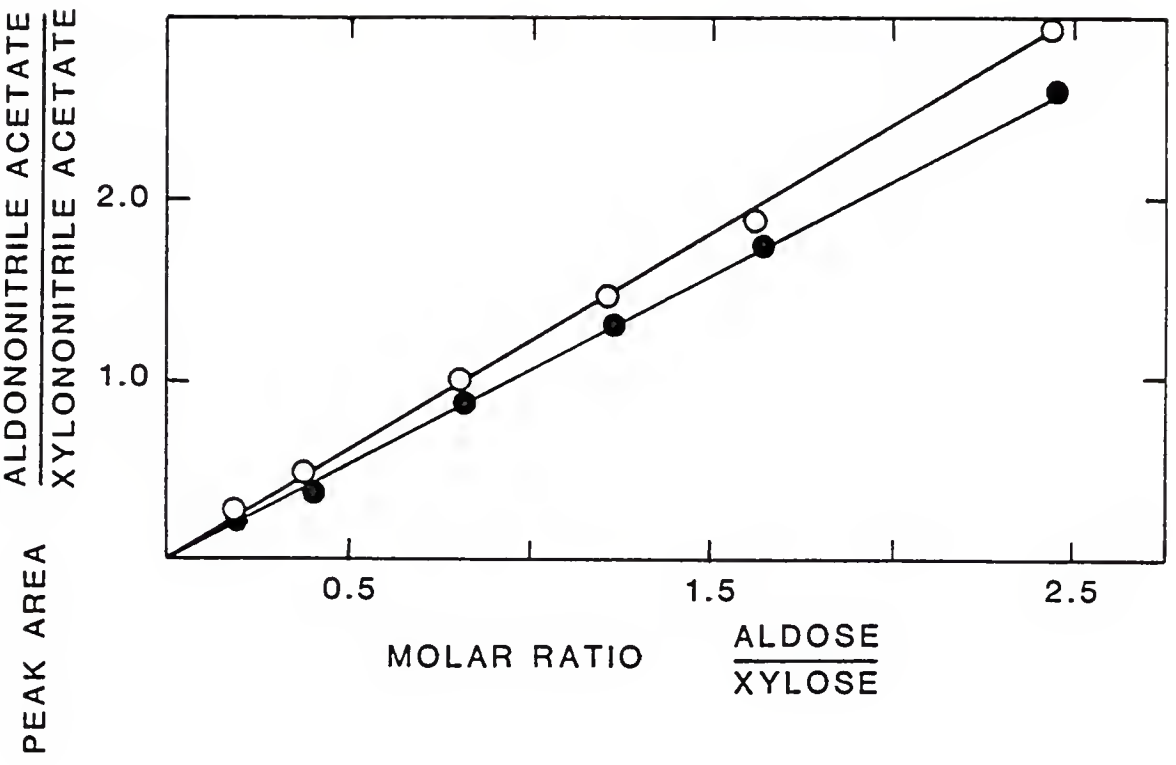
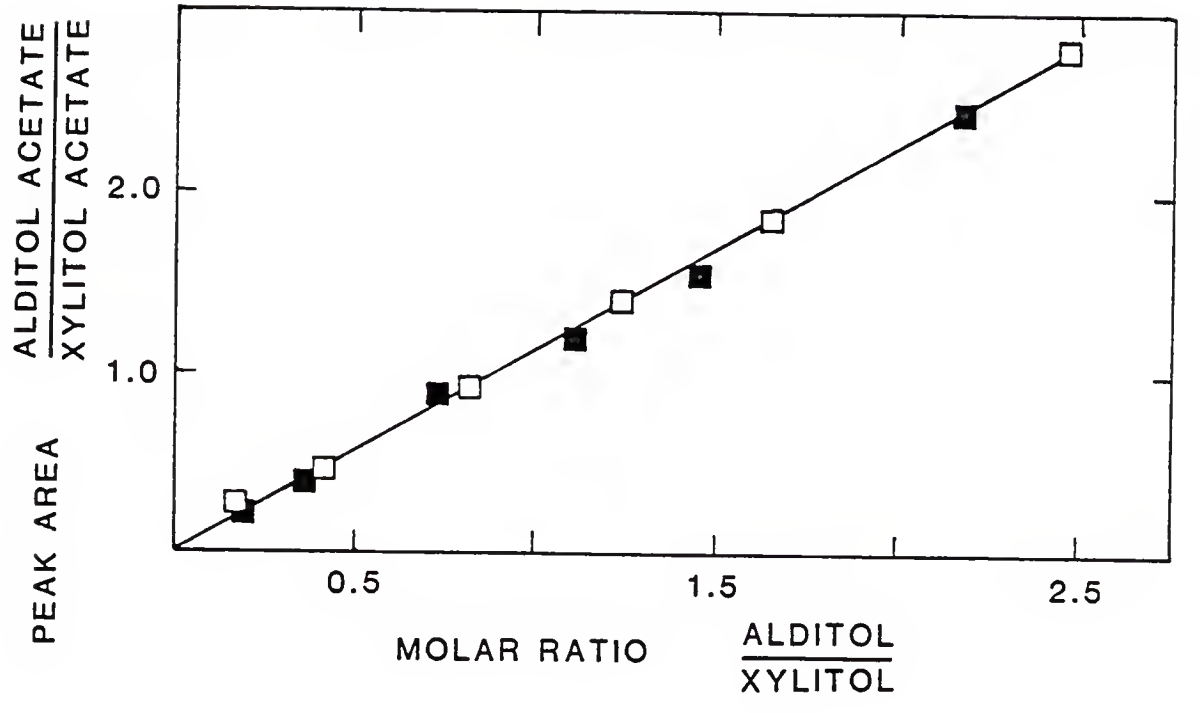

PEAK AREA
ALDONONITRILE ACETATE / XYLONONITRILE ACETATE
2.0
1.0
0.5
1.5
2.5
MOLAR RATIO
ALDOSE / XYLOSE

FIGURE 6

Quantitation of alditols as the peracetylated alditols.

Alditols (mannitol(□), glucitol (■) and xylitol (internal standard)) were converted to the PAAs and the derivatives were separated by gas-liquid chromatography on 1% OV-225 at 190°C as described under "Experimental Procedures". The ratio of peak areas of sample/standard was plotted against the molar ratio of the sample/standard. Correction factors necessary for the calculation of unknowns were determined to be: Mannitol/Xylitol, 0.87; Glucitol/Xylitol, 0.94.


PEAK AREA
ALDITOL ACETATE / XYLITOL ACETATE
2.0
1.0
0.5
1.5
2.5
MOLAR RATIO
ALDITOL / XYLITOL

furanose and pyranose byproducts. The PAANs are more labile than the PAAs; the former partially decompose in the injector of the gas-liquid chromatograph[1]. Observations in this laboratory support these conclusions as we have found that the yield of PAANs is lower than that of the corresponding peracetylated alditols from derivatization of equal amounts of the respective aldoses and alditols. Several small unidentified peaks also eluted after these derivatives and by elution at higher temperatures on 1% OV-225 as was seen by Furneaux. This discrepancy was accounted for, however, by the use of internal standards in each sample, since it was found that the aldoses behaved reproducibly as a group, as do the alditols (Fig. 5 and 6).

Preparation of reduced oligosaccharides--Oligosaccharides O-linked to the protein were released by treating 50-200 mg of each glycoprotein with 0.1N NaOH and 0.3M $NaBH_4$ at 40°C for 48 hours [87]. The reactions were performed in sealed culture tubes with teflon screw caps in a volume of 15 ml. Reaction products were neutralized with 2 N acetic acid using 5 drops of n-octanol to control foaming and taken to dryness on a rotary evaporator at 50°C. The residues were evaporated with methanol several times to remove excess borate and then deionized through a small column (50 ml) of Amberlite MB-3. The eluant was taken to dryness, dissolved in 400 µl of water and injected onto a Biogel P-2 (-400 mesh) column (0.25 in x 8 ft) attached to a Waters high performance liquid chromatograph (HPLC;see below). Oligosaccharides were eluted with water and the eluant stream monitored with a differential refractometer. Oligosaccharides were

[1]Bradbury, A.G.W. (1984) Personal Communication.

purified either by rechromatography of pooled fractions under the same conditions, or by passage through a Partisil PXS 10/25 PAC polar reverse-phase column, also by HPLC. Composition of each oligosaccharide and identification of the sugar attaching the oligosaccharide to the protein was determined following mild acid hydrolysis of each and conversion of the product to a mixture of the alditol and aldononitrile acetates by the PAAN method previously described. Derivatives were then analyzed by gas-liquid chromatography and gas chromatography/mass spectrometry.

Preparation of Methylsulfinyl Carbanion--The methylsulfinyl carbanion suspension for methylation analysis was prepared by an adaptation of the methods of Lindberg [89] and Spiro [90]. Sodium hydride was obtained in a 50% mineral oil dispersion as it reacts explosively with water; all glassware was acid cleaned, baked and then kept over phosphorous pentoxide until use. Under a dry nitrogen stream, 4.75 g of sodium hydride was weighed into a 250 ml erlenmeyer flask, equipped with a two-hole rubber stopper (with a $CaCl_2$ trap and a tube for nitrogen) and a small magnetic stirrer bar. To this was added 100 ml DMSO (dried over 3A molecular seive) to achieve a final concentration of 2M sodium hydride, and the mixture stirred under a nitrogen stream for one hour. Aliquots (25 ml) of the suspension were poured immediately into 30 ml serum bottles, sealed with rubber caps and placed on ice. The suspensions were still generating hydrogen gas; so the caps were punctured with two syringe needles and the bottles flushed with nitrogen until the mixture froze solid. The methylsulfinyl carbanion suspension was stored under nitrogen (with

Drierite) at -20°C. Under these conditions the preparation was good for at least four years. When ready for use, the caps were punctured again with two syringe needles and flushed with nitrogen in a sonicator bath for one hour. Aliquots of the suspension were removed with an 18 gauge needle (smaller needles clogged up with the thick slurry) and the mixture frozen on ice again under a nitrogen stream.

Methylation Analysis--Glycoproteins and oligosaccharides released by reductive ß-elimination were methylated by the method of Hakomori [91] and samples were subsequently hydrolyzed, reduced and acetylated by a method adapted from those of Björndal et al. [92] and Gum [93]. Samples (oligosaccharides, 0.5-1.5 mg; glycoproteins, 10 mg) were first dried over phosphorous pentoxide overnight in 5 ml Reacti-Vials, dissolved in 1 ml dry DMSO and closed with teflon/silicone seals. The seals were punctured with two syringe needles, 1.0 ml of 2M methylsulfonylmethylsodium was injected and the needles removed after flushing the vials with nitrogen. The vials were agitated in an sonicator bath for one hour. After another 2 hours, 1 ml methyl iodide was injected dropwise and the vials sonicated for 30 min. The products were partitioned into $CHCl_3$ (5 ml) and water (7.5 ml) and the water layers washed again with $CHCl_3$ (2 x 3 ml). The organic layers were pooled and repeatedly washed with water (3 x 5 ml). Excess water was removed from the organic layers by addition of a spatula tip of $MgSO_4$ and the supernatant filtered through glass wool into 5 ml Reacti-Vials and evaporated to dryness under nitrogen. Methylated oligosaccharides were then hydrolyzed with 88% formic acid (2 ml) for 2 hours at 100°C. The products were taken to dryness

under nitrogen and the hydrolysis completed with 0.25 M H_2SO_4 (1 ml) at 100°C for 12 hours. Samples were transferred to small test tubes, 0.54 g $BaCO_3$ (10% excess) was added to neutralize the hydrolysates and the supernatants removed after centrifugation (5000 rpm, 10 min). The pellets were extracted washed with 1 ml water, centrifuged again and the supernates pooled. The partially methylated monosaccharides, then in 3 ml of water, were then reduced by addition of 20 mg $NaBH_4$ at room temperature for 2 hours. The supernatants were then taken to dryness in 5 ml Reacti-Vials and evaporated with methanol (3 x 2 ml). After drying overnight with P_2O_5, the partially methylated alditols residues were acetylated with pyridine (0.25 ml) and acetic anhydride (0.25 ml) at 90°C for 1 hour. The reaction mixtures were taken to dryness under nitrogen at 60°C, evaporated with toluene (3 x 1 ml) and dissolved in 20-50 µl $CHCl_3$. Products were analyzed by gas-liquid chromatography on 3% neopentyl glycol succinate, and positively identified by gas chromatography/mass spectrometry (see below).

Tetramethyl and trimethyl alditol acetate standards for GLC and GC/MS comparison were prepared by methylation of either (i) 5 mg of yeast mannan for the mannose series or (ii) 5 mg each of the glucose disaccharides, kojibiose (α1-2), nigerose (α1-3), cellobiose (ß1-4) and gentiobiose (ß1-6). Pentamethyl alditol acetate standards were prepared after reduction of 5 mg of each of the glucose disaccharides described with 20 mg sodium borohydride in 2 ml of water at room temperature overnight. The reactions were stopped with 2 N acetic acid, in the presence of n-octanol, and each sample taken to dryness under reduced pressure. Excess borate was removed by evaporation as

the volatile methyl ester by addition of aliquots of methanol (3 x 10 ml) and the samples subsequently transferred to 5 ml Reacti-Vials and lyophillized in preparation for methylation.

Acetolysis--Reduced tri- and tetrasaccharides were subjected to acetolysis by the method of Tai et al. [94] as modified by Li et al. [95]. Oligosaccharides (100 µg) were dissolved in 0.5 ml pyridine and 0.5 ml acetic anhydride in 5 ml Reacti-Vials and left at room temperature for 70-74 h. The vials were then heated at 80°C for 4 h. Samples were taken to dryness under nitrogen and evaporated with toluene (3 x 1 ml). Acetylated oligosaccharides were then acetolyzed with 1.0 ml acetic anhydride-acetic acid-H_2SO_4 (10:10:1) at 40°C for 16 h. The products were then partitioned into $CHCl_3$ (1 ml) and water (1 ml). The aqueous layer was removed and the organic layer washed repeatedly with water to remove the color (3 x 3 ml). The aqueous washes were then extracted with $CHCl_3$ (3 x 1 ml) and all the $CHCl_3$ fractions pooled and dried. The residue was then deacetylated with 1.0 ml 0.1% sodium methanolate for 30 min at room temperature. The reaction was neutralized with 2.0 ml ethyl acetate and the products evaporated to dryness under nitrogen. The residue was then dissolved in 10-30 µl of water and the products separated by polar reverse-phase HPLC on a Whatman Partisil PXS 10/25 PAC column. The compositions of purified oligosaccharides were then determined by gas-liquid chromatography as the peracetylated alditol and/or aldononitriles (see below).

Glycosidase digestion--Oligosaccharides released from the glycoproteins by reductive ß-elimination were subjected to sequential glycosidase digestion which provided information as to the anomeric nature of each sugar residue and also the sequence of sugars in each oligosaccharide. Samples of each oligosaccharide (100 µg) were placed in 250 µl Microfuge tubes (Beckman Instruments, Inc., Palo Alto, CA) and lyophillized. Incubation with yeast α-glucosidase (maltase) was performed with one unit of enzyme in 0.05 ml of 0.05 M potassium phosphate, pH 6.8, for 12 hours at 40°C with 10 µl of toluene added to ensure sterility. One unit of enzyme was then added every 12 hours for the next 36 hours until four units of enzyme had been added. Incubation with jack bean α-mannosidase was performed with one unit of enzyme in 0.05 ml of 0.05 M sodium citrate, pH 4.5 for 48 hours at 40°C with 10 µl toluene added. Products were separated directly by HPLC on a Partsil PXS 10/25 PAC polar reverse-phase column, equipped with an Bondapak AX/Corasil anion exchange precolumn. Oligosaccharides were subsequently incubated with a different glycosidase enzyme and the resulting monosaccharides were analyzed as the alditol and/or aldononitrile acetates by gas-liquid chromatography.

Gas-Liquid Chromatography--Monosaccharide components of oligosaccharides and glycoproteins were identified and quantified after separation of acetylated derivatives by gas-liquid chromatography and as such it was necessary to achieve a separation of alditol and aldononitrile acetates of component sugars of the glycoproteins (see Fig. 3). Although separation of PAANs [85] and PAAs [86] have been reported, these methods were extensively modified to

permit simultaneous separation of the two classes of sugar derivatives. All gas-liquid chromatography was performed on a Varian Model 2700 gas chromatograph (Varian Associates, Sunnyvale, CA) equipped with a flame ionization detector and attached to an Autolab System 1 computing integrator (Spectra-Physics, Santa Clara, CA). Alditol and aldononitrile acetates were separated on a 1% OV-225 on Chromosorb G H/P (80-100 mesh) glass column (2 mm X 8 ft.), isothermally at 180°C. Partially methylated alditol acetates were separated on a 3% neopentyl glycol succinate on Gas Chrom Q (100-120 mesh) glass column (2 mm x 20 ft.), with isothermal elution at 190°C. The injector and detector temperatures were 230°C and 260°C, respectively, for both columns. Trimethyl alditol acetates were found to be less well resolved on packed columns of 1% OV-225, whether separated isothermally or under a variety of temperature programmed conditions.

Gas Chromatography/Mass Spectrometry--While separations of the derivatized sugars can be performed by gas-liquid chromatography, definitive identification was achieved by this technique in conjunction with mass spectrometry. Two types of fragmentation were used in this work, electron impact (EI) and chemical ionization (CI), and each yields different information regarding the molecules being identified. Mass spectrometry is not normally used for quantitation of samples as a relatively low fraction of the sample is ionized (about 1% for EI) and so all quantitation of derivatives was performed on the gas chromatograph. EI fragmentation is severe as the sample is exposed directly to an electron beam in the ionizing chamber; hence EI spectra

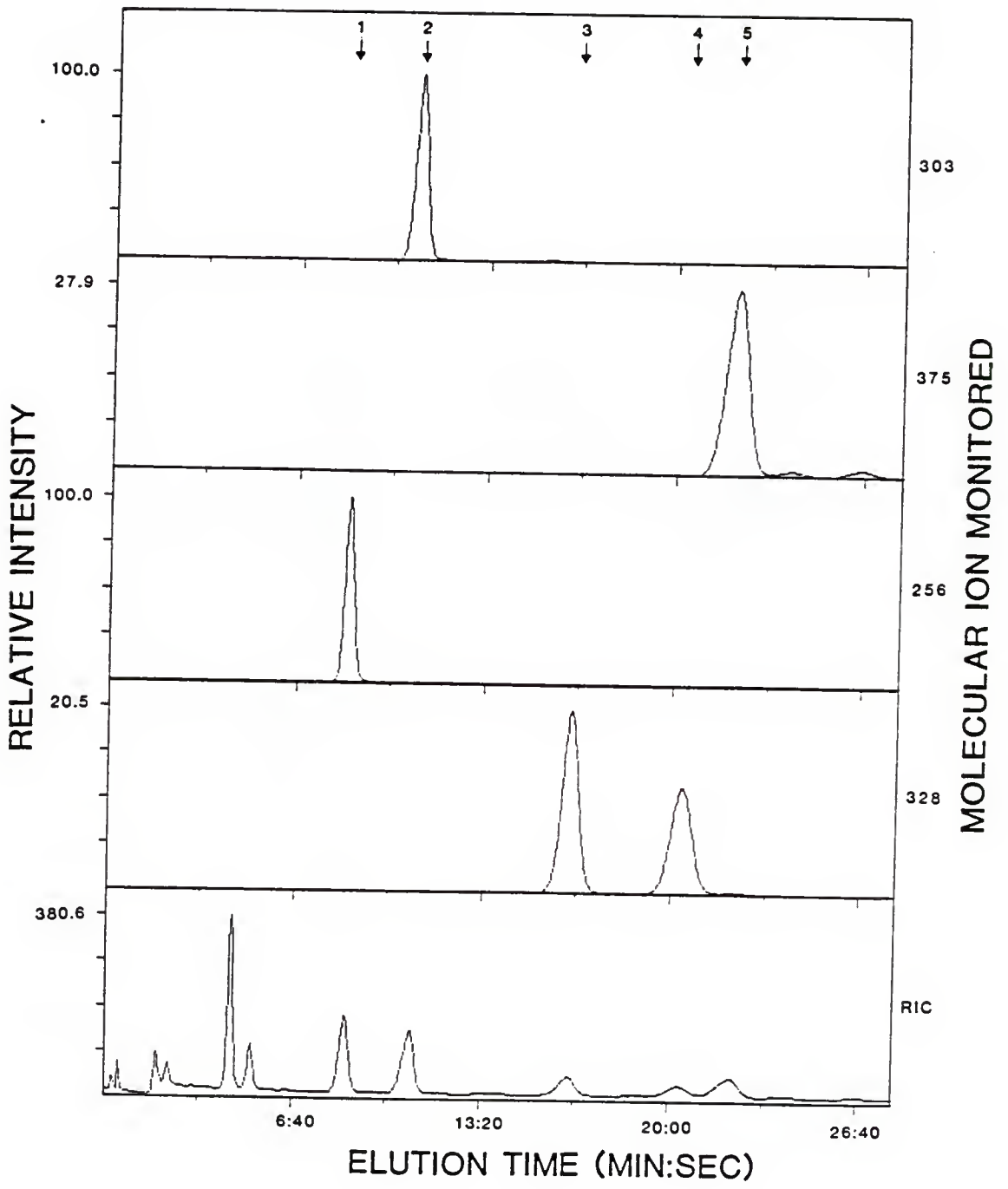
represent an array of structural fragments (fingerprints) of the molecules. It should be mentioned that peracetylated alditol (Appendix A) and peracetylated aldononitrile (Appendix B) derivatives of mannose and glucose fragment identically by EI; hence the need for prior separation by gas-liquid chromatography. This is more important when comparing the partially methylated alditol acetates, where coordinately methylated mannose and glucose derivatives fragment identically. Differentially methylated residues, while having many similar fragments, give rise to unique spectra (see Appendix C).

CI fragmentation is less severe as the ionizing chamber is filled with an excess of reagent gas, i.e. isobutane, and thus it is the gas molecules that are ionized directly. These ionized gas molecules then collide with sample molecules causing a milder fragmentation than that which occurs in EI, as less energy is transferred. CI spectra thus provide very accurate molecular weight information by a monitoring of the molecular ion (M^+), generated by loss of an electron from the molecule during collision. As galactononitrile acetate and mannitol acetate were found to coelute on a packed 1% OV-225 column, mass fragment scanning was useful in ruling out the presence of galactose in glycoprotein samples following reductive ß-elimination and hydrolysis. This is shown in Fig. 7, where the mass ion for galactononitrile acetate is 356 and none was shown to be present under the mannitol acetate peak (M^+=375). CI spectra for peracetylated alditol (Appendix D) and peracetylated aldononitrile (Appendix E) standards are shown. By providing primarily molecular weight information, CI spectra cannot distinguish between the various trimethyl alditol acetates prepared as standards but of

FIGURE 7

Molecular ion scanning of peracetylated alditol and aldononitrile derivatives of monosaccharides released from cellobiohydrolase I(D) following reductive ß-elimination and subsequent acid hydrolysis.

Molecular ions were selected out of the profile following chemical ionization gas chromatography/mass spectrometry. Scanned were the M^+ ions for: pentitol acetates (303), hexitol acetates (375), pentononitrile acetates (256) and hexononitrile acetates (328). The bottom panel represents the reconstituted ion current (RIC), or the total material in the sample. Elution times are shown for peracetylated 1. xylononitrile; 2. xylitol; 3. mannononitrile; 4. gluconononitrile; 5. mannitol.


1
2
3
4
5
100.0
303
27.9
375
100.0
256
20.5
328
380.6
RIC
RELATIVE INTENSITY
MOLECULAR ION MONITORED
6:40
13:20
20:00
26:40
ELUTION TIME (MIN:SEC)

course can distinguish between the tri- and tetramethyl alditol acetates (see Appendix F).

Gas chromatography/mass spectrometry was performed either on a Finnegan 4021 GC/MS/DS or on a Finnegan TSQ GC/MS/MS/DS instrument. All chromatographic conditions were the same as those for gas-liquid chromatography except the 3% neopentyl glycol succinate column was only 8 ft long. Electron impact spectra were recorded at an ionizing potential of 70 eV with an ion source temperature of 300°C. Chemical ionization spectra were recorded at an ionizing potential of 55eV and a filament current of 0.3A, using isobutane as the reagent gas at 5 x 10^{-5} torr.

High Performance Liquid Chromatography--HPLC separations were performed with a Waters Model 6000 pump, Model U6K injector and Model R401 differential refractometer (Waters Associates, Inc., Milford, MA). Analytical separations were performed by an adaptation of the method of Gum and Brown [96], on a Partisil PXS 10/25 PAC polar reverse-phase column (Whatman Inc., Clifton, NJ) equipped with a Bondapak AX/Corasil anion exchange precolumn (Waters Associates). Elution was performed at a flow rate of 1.5 ml/min at room temperature, with either 75% or 77% acetonitrile in water and at a pressure of 1400 psi. Preparative separations were performed on a water-jacketed Biogel P-2 (-400 mesh) gel filtration column (0.25 in x 8 ft), with no precolumn, using a Haake Constant Temperature Circulator. Oligosaccharides were eluted with water at 60°C at a flow rate of 0.7 ml/min and at a pressure of 700 psi.

NMR Analyses--NMR analyses were performed on both glycoproteins and on oligosaccharides isolated from them after preparative reductive ß-elimination and the spectra generated provided information about the entire molecule being analyzed. For ^{1}H-NMR, analysis of the glycoprotein is not practical due to the excessive number of signals generated. For the oligosaccharides, the ^{1}H-NMR provided information as to the number of anomeric residues present and also, in the case of glucose, the nature of the anomeric linkage. For mannose, the splitting of the anomeric proton for both α- and ß-anomers is less than 2 Hz, which is below the resolution of the instrument at 300 MHz.

Characterization of oligosaccharides attached to the glycoproteins was possible by proton coupled and proton decoupled ^{13}C-NMR as these signals (between 60-110 ppm) were far removed from those of amino acid residues. The decoupled spectra generated signals for each carbon nucleus in the sample, although due to the closeness of many of the signals, it was not possible to assign all of them. The signals generated by the anomeric carbons, located between 98-106 ppm, were distinct and were far downfield from the other carbohydrate signals. These can be assigned, on the basis of literature values and by analysis of the compounds by chemical methods. The number of hydroxymethyl carbons can also be determined as they generate signals at the upfield end of the carbohydrate region between 62 and 66 ppm. But like the ^{1}H-NMR, decoupled ^{13}C-NMR spectra are a characteristic fingerprint of the molecule, even though it is not possible to assign all the signals. Coupled ^{13}C-NMR, like ^{1}H-NMR, provided information as to the anomeric nature of the sugar residues

in the oligosaccharides. The coupling constants generated from the anomeric carbons were characteristic of either α-linkages (170 Hz) or ß-linkages (160 Hz), although the resolution of signals in coupled spectra is much poorer than those acquired in the decoupled mode.

CBH I(D) was prepared by dialyzing against water, lyophillizing and dissolving the protein into D_2O (98.5%). CBH II was less soluble and counterions were needed, so the protein was dialyzed against 50 mM NaCl and made to at least 60% D_2O. The endoglucanases were prepared in the same way, except that 20 mM NaCl was used. The reduced oligosaccharides were lyophillized and picked up in 100% D_2O several times for all the NMR analyses.

^{13}C-NMR spectra were obtained on a Nicolet NMR Spectrometer with a 70.5 kG field and a broadband tunable probe operating in the Fourier Transform mode at 75.46 MHz. D_2O was used as solvent in all samples and the field was locked on the deuterium signal. Spectra were collected with broad band proton decoupling either on or off, as indicated in the particular experiment. Pulse widths and individual recycle times used were 29.00 µsec and 3.00 msec, respectively, and in all experiments, the post acquisition delay time was greater than $5T_1$. The spectra were filtered to improve the signal to noise ratio and this treatment broadened the lines by 1 Hz. Chemical shifts are reported in parts per million (ppm) from internal (trimethylsilane)-1-propane sulfonate (TSP). Under these conditions, the anomeric carbon of ß-D-glucopyranose resonates at 98.76 ppm.

^{1}H-NMR spectra were obtained on the same instrument with a fixed frequency (300 MHz) probe and spectra were acquired at both

25°C and 70°C to eliminate the possibility of signals "hidden" under the large HOD signal (4.77 ppm at 25°C).

Amino Acid Analysis--One milligram samples of the glycoproteins were ß-eliminated with 0.1M NaOH and 0.3M $NaBH_4$ for 48 hours at 40°C. The resultant unsaturated amino acids were subsequently reduced with palladium chloride and polypeptides hydrolyzed to the free amino acids as described by Downs et al. [97]. Reactions were performed in sealed screw-cap culture tubes in a volume of 2.0 ml. After alkaline treatment, 5 drops of n-octanol and a small stirrer bar were added. One milliliter 0.8M HCl was added followed by 0.1 ml 0.08M $PdCl_2$ and the solutions mixed. Two milliliters $NaBH_4$ (0.66M in 0.1M NaOH) and 2.0 ml $PdCl_2$ (0.016M in 0.8M HCl) were then added simultaneously in a dropwise fashion, while mixing rapidly. The total volume was now 7.1 ml and to this was added an equal volume of concentrated HCl. The tubes were resealed with teflon liners and heated at 110°C for 24 h. Each sample was cooled, transferred quantitatively to a 100 ml round bottom flask and taken to dryness on a rotary evaporator at 50°C. Residual HCl was removed by repeated evaporation with water (3 x 10 ml). The final volume was carefully dissolved in 1 ml lithium citrate buffer (0.2M, pH 2.2) and transferred to a 5 ml volumetric flask. The round bottom flask was then rinsed repeatedly (4 x 1 ml) and the washings added to the 5 ml volumetric flask and mixed. Samples containing $PdCl_2$ contained a small amount of black particulate precipitate, which was allowed to settle before analysis. Aliquots (0.1 ml) were injected onto a Beckman Model 119CL amino acid analyzer (Beckman Instruments, Inc., Palo Alto, CA) in the physiological column mode. α-Aminobutyric acid (product of

threonine ß-elimination and reduction) was detected with ninhydrin [98] and eluted between 76-77 minutes. The peak was integrated and compared to an internal standard, α-aminoguanidinopropionic acid. Controls were performed on (i) samples which had undergone $PdCl_2$ treatment without previous ß-elimination, to determine if any background α-aminobutyric acid was produced and (i) on samples which had undergone neither $PdCl_2$ treatment nor ß-elimination to determine any possible effects of these treatments on any amino acids released by hydrolysis.

Antibody Preparation and Immunochemical Analysis--Antisera to CBH I(D), CBH II and the endoglucanases were prepared in New Zealand White rabbits by a method adapted from that of Hurn and Chantler [99]. Samples of protein (2.5-3.0 mg) which had been dialyzed to water and lyophillized were dissolved in 1 ml water and emulsified with 3.5 ml Freunds Complete Adjuvent. Each rabbit, 1-2 Kg in size, was injected with a total of 2 ml of one of the proteins, 1 ml into each thigh muscle. Booster injections were given three weeks later under the same conditions. Animals were test bled periodically and each antisera titer determined by immunodiffusion on agar against all the cellobiohydrolases and endoglucanases to check for cross-reactivity. Rabbits were then sacrificed after seven weeks, 70-100 ml of whole blood collected from each and serum prepared after removal of whole cells by centrifugation.

RESULTS AND DISCUSSION

Cellobiohydrolase I(D)

Molecular Properties of CBH I(D)--CBH I(D) has been shown to constitute almost 60% of the extracellular protein secreted by Trichoderma reesei QM 9414 grown on cellulose. This protein has also been found to have a molecular weight of 54,000 daltons, as determined by sedimentation equilibrium and amino acid analysis [49]. CBH I(D) was obtained in highly purified form here after elution from DEAE-Sephadex at pH 3.6, as determined by native polyacrylamide gel electrophoresis (Figs. 1 and 8). Horizontal isoelectric focusing of this material gave rise to a single band which was isoelectric at pH 4.14 $\pm$ 0.06. Antisera produced against CBH I(D) in rabbits generated a single precipitin band after immunodiffusion against the homologous protein, but had no cross-reactivity at all to CBH II or to any of the endoglucanases tested. An experimentally determined extinction coefficient of 13.80 $\pm$ 0.20 (for a 1% solution at 280 nm) was used in each calculation and pure CBH I(D) was found to have an A_{280}/A_{260} ratio of 1.82-1.84.

Neutral Carbohydrate Composition--The carbohydrate was removed from CBH I(D) either by reductive ß-elimination to release O-linked oligosaccharides or by mild acid hydrolysis to release all sugars as

FIGURE 8

Polyacrylamide disc gel electrophoresis of crude extracellular protein prepared from T. reesei QM 9414 and highly purified cellobiohydrolase I(D).

Lanes 1 and 2 contain 100 µg each of crude extracellular protein that was applied to the DEAE-Sephadex column. Lanes 3 and 4 represent 40 µg each of highly purified CBH I(D) (from a pool of fractions 600-620) eluted from DEAE-Sephadex at pH 3.6 (see Fig. 1). Lanes 1 and 3 were stained with Coomassie Blue for protein and lanes 2 and 4 were stained with the periodic acid-Schiff reagent for carbohydrate.


1
2
3
4

monosaccharides. The products were converted to the respective peracetylated alditols and aldononitriles and analyzed by gas-liquid chromatography (see Table II). The protein was found to contain about 13 mannosyl and 5 glucosyl residues which account for 6.0% of the 54 Kdalton molecular weight. This composition agrees well with previous data (Table I,[49]), even though protein was determined by the Lowry colorimetric assay and not the extinction coefficient, as in the case of the present work. This also agrees well with a value of 5.8 weight percent carbohydrate obtained from phenol-sulfuric acid determination using mannose as a standard.

Analysis of the products released by reductive ß-elimination as both the alditol acetates (derived from sugars directly attached to the protein) and aldononitrile acetates provided information as to the total carbohydrate composition, the number of O-linked attachment sites to the protein and the type of sugar attached (Table II). For CBH I(D), mannose (identified as peracetylated mannitol) was the only O-linked saccharide found to be attached to the protein and at an average of 5.9 $\pm$ 0.6 sites. The fact that the total number of neutral sugars recovered from either reductive ß-elimination or acid hydrolysis were approximately the same suggests that all the neutral carbohydrate was alkali-labile (i.e. O-linked). This ruled out the possibility of large N-linked structures, common in many glycoproteins, as they are stable under the mild alkaline conditions used.

O-linked Oligosaccharides--O-linked oligosaccharides released from CBH I(D) by reductive ß-elimination were separated by

TABLE II

NEUTRAL CARBOHYDRATE COMPOSITION OF CELLOBIOHYDROLASE I(D)

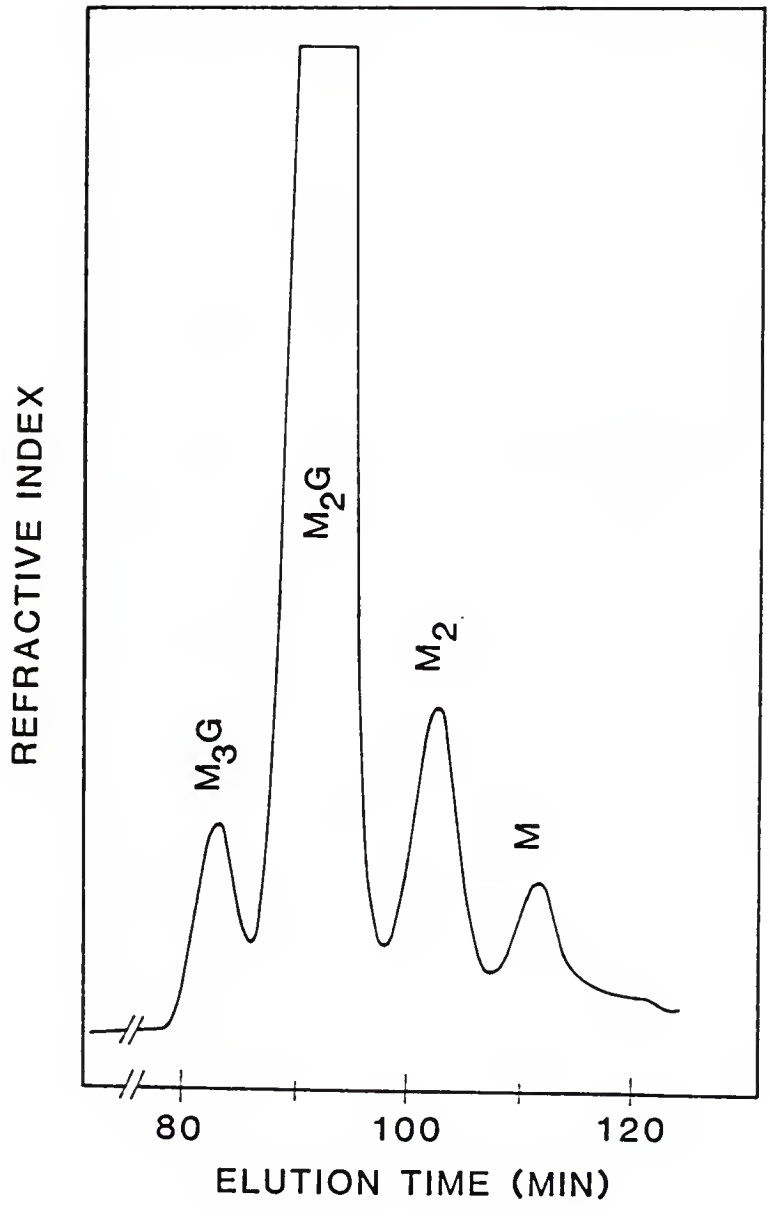
CBH I(D) was treated with either mild acid or alkaline borohydride and the products analyzed either as a mixture of PAANs and PAAs or, after reduction with sodium borohydride, as the PAAs. For further details , see "Experimental Procedures." Values are expressed in moles/mole protein.

Peracetylated Alditols and Aldononitriles	Method of Carbohydrate Release	
	Acid Hydrolysis	Alkaline Borohydride
Mannononitrile	15.2 ± 1.0	6.2 ± 0.6
Gluconononitrile	5.9 ± 0.5	5.2 ± 0.8
Mannitol		5.9 ± 0.6
Mannitol	12.8 ± 0.5	13.8 ± 0.5
Glucitol	5.3 ± 0.5	4.9 ± 0.3

FIGURE 9

Separation on a Biogel P-2 column of oligosaccharides released from cellobiohydrolase I(D) by reductive ß-elimination.

200 mg CBH I(D) was ß-eliminated with 0.1M NaOH and 0.3 M $NaBH_4$ at 40°C for 48 hours. The reaction was stopped with 2N acetic acid and the mixture taken to dryness. Following methanol evaporation, the reduced oligosaccharides were deionized over Amberlite MB-3, lyophillized and then applied to a Biogel P-2 column. For further details see "Experimental Procedures". Peaks labelled M, M_2, M_2G and M_3G correspond to Mono-, di-, tri- and tetrasaccharides, respectively.


REFRACTIVE INDEX
M3G
M2G
M2
M
80
100
120
ELUTION TIME (MIN)

gel filtration on Biogel P-2 (Fig. 9), purified by rechromatography on the same column and weighed (Table III). Analysis of the eluate permitted an estimate of an average of 4.2 chains of trisaccharide, 1.1-1.2 of the di- and monosaccharides and 0.7 chains of tetrasaccharide per molecule of glycoprotein; calculations were made on the assumption that each oligosaccharide chain has one attachment site to the protein. These account for about 7.2 oligosaccharide chains O-linked to CBH I(D) which is in fair agreement with an estimate of 5.9 $\pm$ 0.6 attachment sites obtained by gas chromatography data (Table II). The 5.9% total neutral carbohydrate found also agrees well with previous evidence from gas chromatography (6%) and colorimetric determination (5.8%).

Analysis of the composition of each of the oligosaccharides by gas-liquid chromatography (see Table IV) provided the first evidence that these may be a related series. All contain mannose (as mannitol) at the reducing terminus, and the tri- and tetrasaccharides were also found to contain one residue of glucose. Since the peak shapes generated from Biogel P-2 separation were symmetrical and the stoichiometry of the gas chromatographic analysis of the di- and trisaccharides yielded nearly integral values for each residue indicating that each oligomer comprises one species, and is not, therefore, a heterogeneous population. These results further demonstrated the usefulness of the mixed alditol/aldononitrile acetate technique in determining not only the linking sugars, but also the composition of each oligosaccharide.

TABLE III

OLIGOSACCHARIDES RELEASED BY PREPARATIVE
ß-ELIMINATION OF CELLOBIOHYDROLASE I(D)

Oligosaccharides released by reductive ß-elimination of CBH I(D) were separated on Biogel P-2 (see Fig. 9), pooled and lyophillized. Samples were then dried over P_2O_5 and weighed. Calculations were made assuming a molecular weight for CBH I(D) of 54,000 daltons [49].

Oligosaccharide	Weight Percent of Carbohydrate	Weight Percent of Glycoprotein	Moles per Mole CBH I(D)
Tetra-	13.8	0.8	0.7
Tri-	66.9	4.0	4.2
Di-	12.1	0.7	1.1
Mono-	7.1	0.4	1.2
		5.9%	7.2

TABLE IV

COMPOSITION OF OLIGOSACCHARIDES RELEASED FROM CELLOBIOHYDROLASE I(D) BY REDUCTIVE ß-ELIMINATION

Reduced oligosaccharides, purified on Biogel P-2, were hydrolyzed with mild acid and the products analyzed by gas-liquid chromatography as a mixture of the alditol and aldononitrile acetates. For further details, see "Experimental Procedures".

Peracetylated Derivative	Saccharide			
	Mono-	Di-	Tri-	Tetra-
Mannitol[a]	1.0	1.0	1.0	1.0
Mannononitrile	-	1.2	1.1	1.5
Gluconononitrile	-	-	1.2	0.9

[a] mannitol hexaacetate has been normalized to 1.0 assuming one reducing end per oligosaccharide chain prior to ß-elimination.

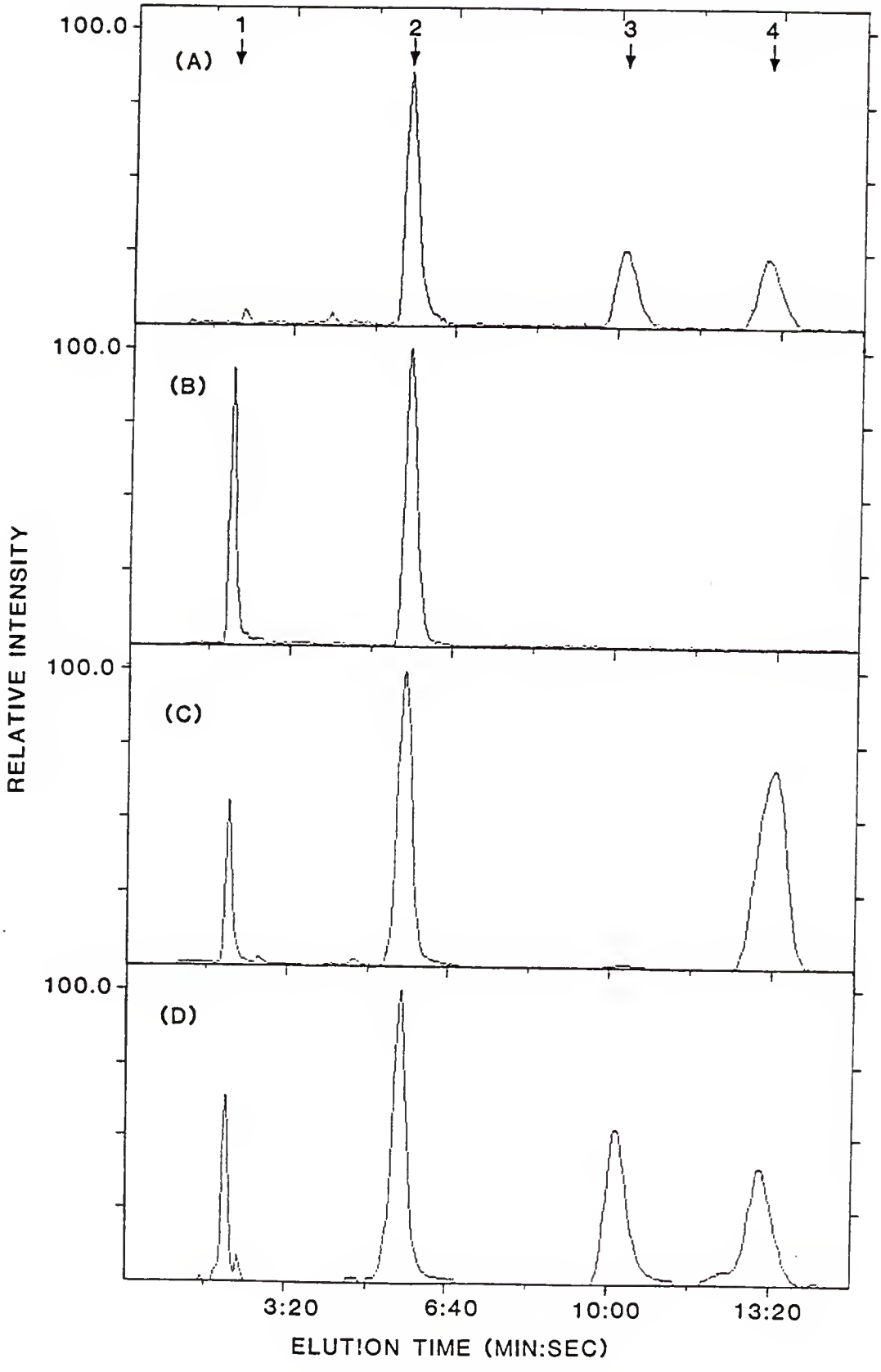
Methylation Analysis--The types of linkages between the sugar residues in the oligosaccharides attached to CBH I(D) were studied by methylation analysis. The partially methylated alditol acetates generated were subsequently separated and identified by gas chromatography/mass spectrometry (see Fig. 10).

The intact glycoprotein yielded three methylated species corresponding to the 2,3,4,6-tetramethyl, 3,4,6-trimethyl and 2,3,4-trimethyl alditol acetates which indicated the presence of non-reducing terminal, 2-substituted and 6-substituted hexoses, respectively (Fig. 10,Panel A). No dimethyl hexitol acetates were observed indicating that all oligosaccharide chains are unbranched. The ratio of the 3,4,6-/2,3,4-trimethyl species is 0.85, which agrees well with the ratio expected (0.83) from previously determined amounts of tri- and disaccharide attached to CBH I(D) (see Table III). The disaccharide yielded two partially methylated alditol acetates corresponding to the 1,3,4,5,6-pentamethyl and 2,3,4,6-tetramethyl species indicating a 1-2 linked mannobiitol (Fig. 10,Panel B). Analysis of the reduced trisaccharide also reveals three peaks corresponding to the 1,3,4,5,6-pentamethyl, 2,3,4,6-tetramethyl and 2,3,4-trimethyl hexitol acetates (Fig. 10,Panel C); these indicated the presence of 2-substituted (reducing end), non-reducing end and 6-substituted residues, respectively. The tetrasaccharide generated four peaks corresponding to the 1,3,4,5,6-pentamethyl, 2,3,4,6-tetramethyl, 3,4,6- and 2,3,4-trimethyl hexitol acetates (Fig. 10,Panel D); these would be expected to arise from 2-substituted alcohol (reducing-end), non-reducing end, 2-substituted and 6-substituted residues, respectively. The absence of the formation of

FIGURE 10

Methylation analyses of cellobiohydrolase I(D) and the oligosaccharides released from cellobiohydrolase I(D) by reductive β-elimination.

G.C./M.S. profiles of partially methylated alditol acetates generated from (A) CBH I(D) (B) reduced disaccharide, (C) reduced trisaccharide and (D) reduced tetrasaccharide. Individual peaks were positively identified by their electron-impact spectra compared with prepared standards for further details, see "Experimental Procedures". Arrows indicate elution of standards: 1. 2-O-acetyl-1,3,4,5,6-penta-O-methyl glucitol; 2. 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl mannitol; 3. 1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl mannitol; 4. 1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl mannitol.


100.0
1
2
3
4
(A)
100.0
(B)
100.0
(C)
100.0
(D)
RELATIVE INTENSITY
3:20
6:40
10:00
13:20
ELUTION TIME (MIN:SEC)

dimethyl species from any of the oligosaccharides suggested that none of the chains are branched.

Electron impact spectra of all the partially methylated alditol acetates were compared with those prepared standards (Appendix C) and examples of those from the tetrasaccharide (Fig. 10,Panel D) are shown (Fig. 11). Glucitol and mannitol peracetates methylated in the same positions coeluted on 1% OV-225 and also gave identical electron-impact fragmentation patterns (data not shown). The methylation profiles, in conjunction with the evidence from glycosidase digestions (see below), suggest a series of related oligosaccharides corresponding to Man(1-2)mannitol and Glc(1-6)Man(1-2)mannitol for the di- and trisaccharides, respectively. The data for the tetrasaccharide can be interpreted as either a Man(1-2)Glc(1-6)Man(1-2)mannitol or a Man(1-6)Glc(1-2)Man(1-2)mannitol oligosaccharide. Evidence from acetolysis (shown below) will support the former structure.

Glycosidase Digestions--Oligosaccharides released from CBH I(D) by reductive ß-elimination were subjected to sequential glycosidase digestion to determine the sequence of residues and the anomeric nature of glycosidic bonds (see Fig. 12). Each of the glycosidases were assayed for contaminating glycosidase activity using the appropriate p-nitrophenyl glycosides. α-Glucosidase from yeast was found to have minor levels of other glycosidase activities (expressed as percent of α-glucosidase activity) as follows: ß-glucosidase (7.9×10^{-5}%), α-mannosidase (5.6×10^{-4}%) and ß-mannosidase (4.7×10^{-5}%). The α-mannosidase from jack bean also

FIGURE 11

Comparison of electron impact spectra obtained from partially methylated alditol acetates of the tetrasaccharide from cellobiohydrolase I(D) with standards.

Panels (A), (B), (C) and (D) are spectra taken from the four peaks in Figure 6, Panel (D). Panel (E) is a standard pentamethyl alditol acetate from derivatization of kojibiitol, and represents 2-O-acetyl-1,3,4,5,6-penta-O-methyl glucitol. Panels (F), (G) and (H) are standards taken from the partially methylated alditol acetates of yeast mannan, and represent 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl mannitol, 1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl mannitol and 1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl mannitol, respectively. The fragmentation pattern of the pairs (A) and (E), (B) and (F), (C) and (G), (D) and (H) were compared for positive identification.

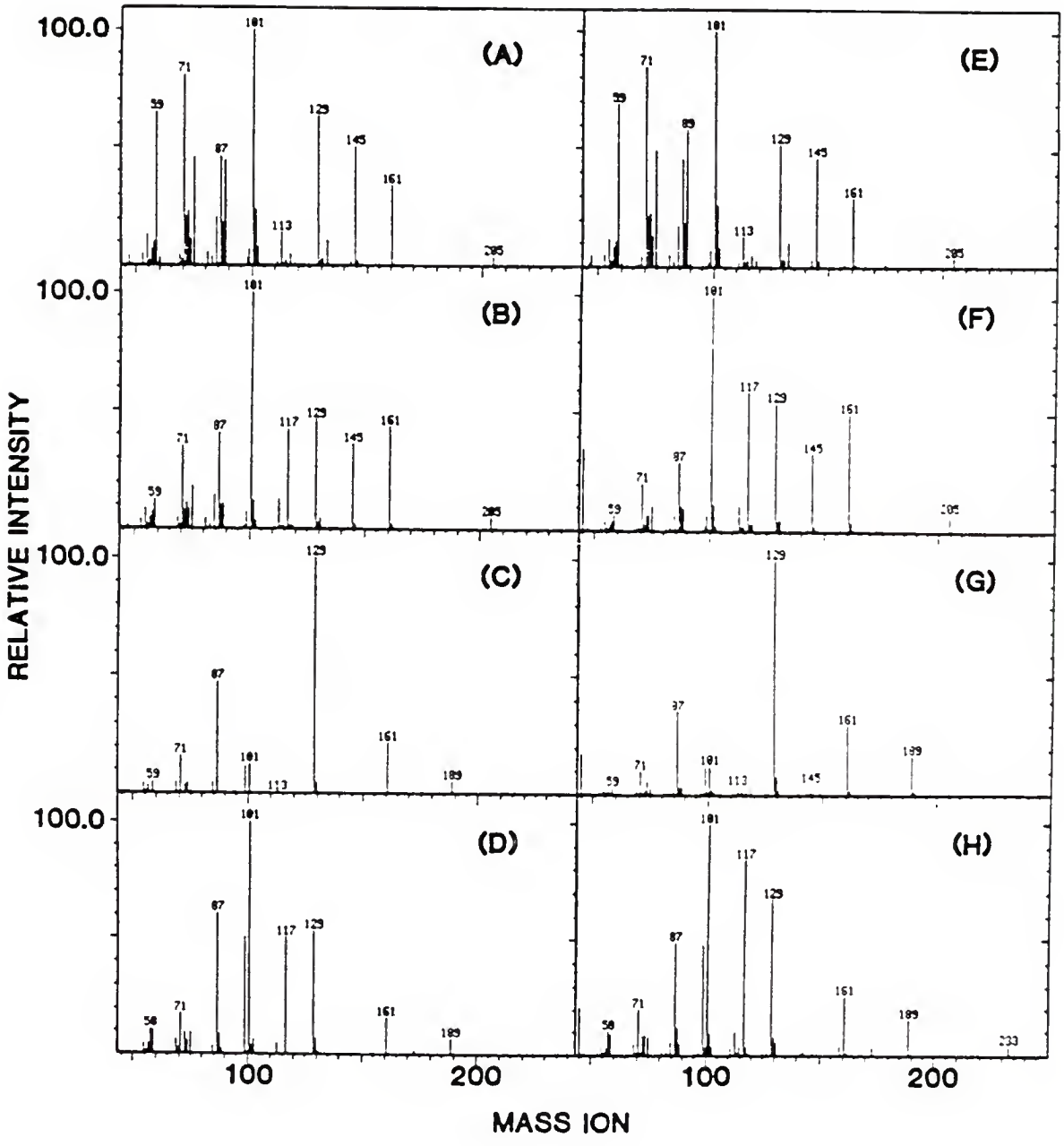
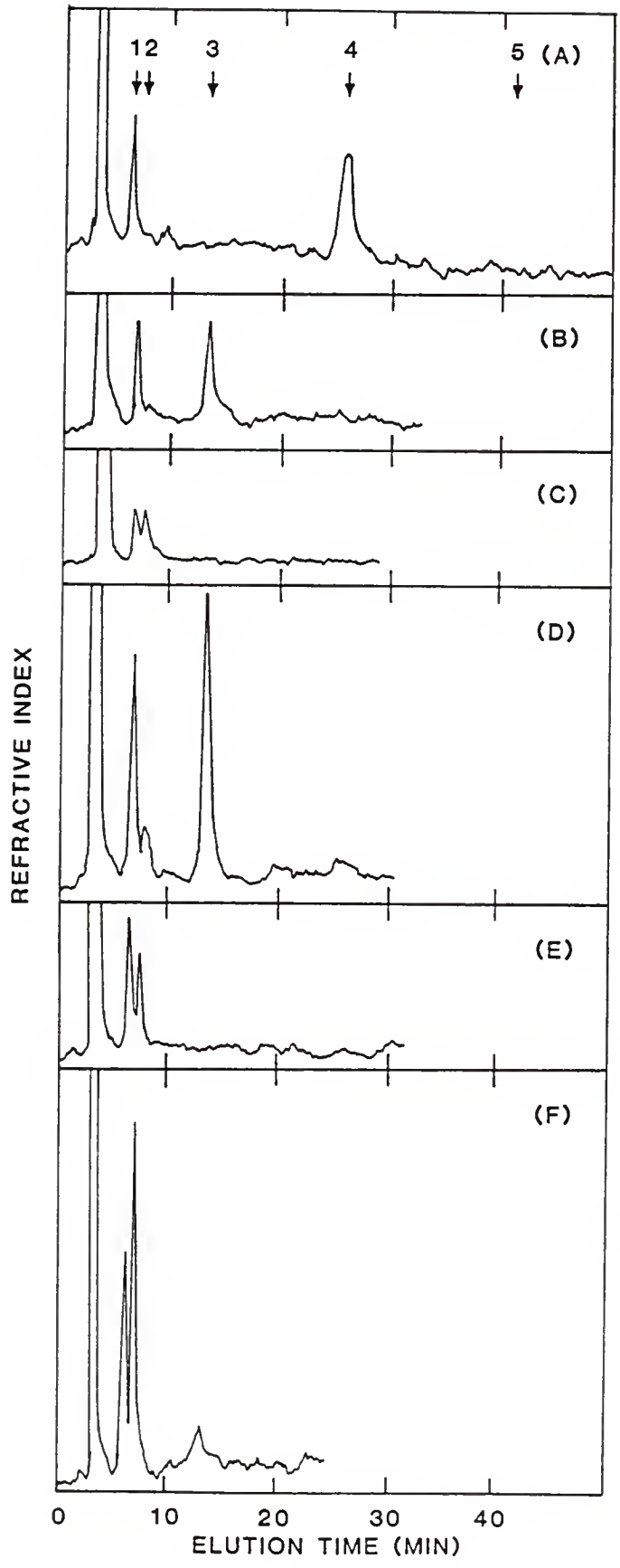

100.0
(A)
(E)
100.0
(B)
(F)
100.0
(C)
(G)
100.0
(D)
(H)
RELATIVE INTENSITY
100
200
100
200
MASS ION

FIGURE 12

HPLC separation of the products of sequential glycosidase digestion of oligosaccharides released from cellobiohydrolase I(D) by reductive ß-elimination.

Shown are products of reduced oligosaccharides from (A) CBH I(D) tetrasaccharide + α-mannosidase, (B) trisaccharide from (A) + α-glucosidase, (C) disaccharide from (B) + α-mannosidase, (D) CBH I(D) trisaccharide + α-glucosidase, (E) disaccharide from (D) + α-mannosidase and (F) CBH I(D) disaccharide + α-mannosidase. Elution times of standards are shown: 1. mannose (glucose); 2. mannitol; 3. CBH I(D) reduced disaccharide; 4. CBH I(D) reduced trisaccharide; 5. CBH I(D) reduced tetrasaccharide. Separations were performed as described in "Experimental Procedures".


12
3
4
5
(A)
(B)
(C)
(D)
(E)
(F)
REFRACTIVE INDEX
0
10
20
30
40
ELUTION TIME (MIN)

was found to have minor contaminating activities of ß-glucosidase (5.8×10^{-4}%), ß-mannosidase (2.2×10^{-3}%) and α-glucosidase (2.8×10^{-4}%). These levels of contaminating activities are so low that they would not be expected to be a substantial influence, unless the incubations were for a long period of time and/or with large amounts of enzyme.

The products of digestion were separated by polar reverse-phase HPLC. The monosaccharides released were converted to peracetylated alditols and/or aldononitriles and were identified by gas-liquid chromatography; subsequently the oligosaccharides were degraded with an alternate glycosidase. All monosaccharides released with α-glucosidase (Fig. 12,Panels B and D) were identified as the peracetylated gluconononitrile. The trisaccharide(s) was not a good substrate for the yeast α-glucosidase and so more units of this enzyme were necessary than in the case of the α-mannosidase. Consequently, small amounts of mannose and mannitol were produced by the minor α-mannosidase contamination. It should be mentioned that digestion of the trisaccharide with ß-glucosidase from Trichoderma did not cause the release of glucose, indicating that the non-reducing terminal glucose residue is not ß-linked. All non-reducing terminal mannose residues, however, were readily cleaved by α-mannosidase (Fig. 12,Panels A,C,E and F) and were identified as the mannononitrile acetates. Recovery of the products after each digestion and the HPLC chromatography step was about 85% in each case.

From the HPLC profiles, it was evident that all the glycosidic linkages are in the α-configuration. The sequence of the tetra- (Fig. 12,Panels A, B and C), tri- (Fig. 12,Panels D and E) and disaccharides

(Fig. 12,Panel F) was proposed to be Manαα1Glcα1Manα1mannitol, Glcα1Manα1mannitol and Manα1mannitol, respectively.

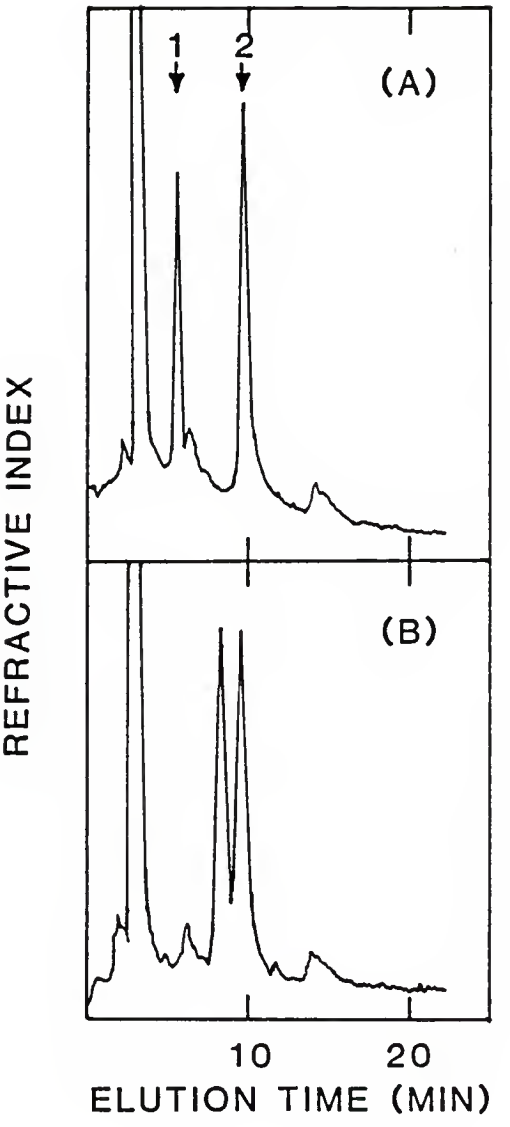
Acetolysis--The reduced tri- and tetrasaccharide from CBH I(D) were each subjected to acetolysis in order to confirm the position of (1-6) glycosidic linkages within the oligosaccharide chains (see Experimental Procedures). After acetylation, oligosaccharides have been shown to be differentially susceptible to hydrolytic cleavage depending on the nature of the glycosidic linkages. The rate of cleavage of acetylated α-linked mannooligosaccharides has been shown to be (1-6)>>(1-3)>(1-2) [71], thus under controlled conditions, (1-6) glycosidic bonds may be hydrolyzed preferentially.

The products of the reaction were separated by HPLC (see Fig. 13) and then further analyzed as the peracetylated alditols and/or aldononitriles by gas-liquid chromatography. The trisaccharide was cleaved into two products, a monosaccharide coeluting with a glucose standard and reduced disaccharide coeluting with authentic reduced disaccharide from CBH I(D) (Fig. 13,Panel A). The monosaccharide was converted to the peracetylated gluconononitrile and the disaccharide into a 1:1 mixture of peracetylated mannitol and peracetylated mannononitrile. This array of products from acetolysis is consistent with a terminal glucose attached by a (1-6) glycosidic linkage to mannobiitol. The tetrasaccharide also was cleaved into two products, a reduced disaccharide which coeluted during HPLC analysis into authentic reduced disaccharide from CBH I(D) and a (unreduced) disaccharide (Fig. 13,Panel B). The reduced disaccharide was converted by hydrolysis into a 1:1 mixture of peracetylated mannitol

FIGURE 13

HPLC separation of the products of acetolysis of oligosaccharides released from cellobiohydrolase I(D) by reductive ß-elimination.

(A) Reduced trisaccharide and (B) reduced tetrasaccharide were subjected to acetolysis as described under "Experimental Procedures". 1 and 2 refer to the elution times of glucose and reduced disaccharide from CBH I(D), respectively. Separations were performed on a Whatman Partisil PXS 10/25 PAC column eluted with 75% acetonitrile at a flow rate of 1.5 ml/min.


1
2
(A)
(B)
REFRACTIVE INDEX
10
20
ELUTION TIME (MIN)

and peracetylated mannononitrile and the disaccharide was converted to a 1:1 mixture of peracetylated mannononitrile and peracetylated gluconononitrile. This acetolysis cleavage pattern is consistent with a (mannose,glucose) disaccharide attached by a (1-6) glycosidic bond to mannobiitol.

^{13}C-NMR--Proton decoupled ^{13}C-NMR at 75.5 MHz was performed on CBH I(D) and the reduced tri- and disaccharides released from CBH I(D) with alkaline borohydride (Fig. 14 and Table V). CBH I(D) generated three signals in the anomeric region (C_1) at 105.1, 102.5 and 100.8 ppm, which indicates the presence of three predominant types of hexoses (Fig. 14,Panel A). It should be mentioned that at this resolution, minor species were not distinguishable, so it is assumed that these signals reflect the trisaccharide (major species) attached to the protein. The signal at 100.8 ppm has been assigned to the glucosyl residue (C-1") at the non-reducing end. The signal at 105.1 ppm was assigned to the central mannosyl residue (C-1') of the trisaccharide and, although the signal is far down field from any known standards, it is suggested that this is due to the unique nature of the substitution of this residue. By comparison with published values [100], the signal at 102.5 ppm was assigned to a mannosyl residue (C-1) α-linked to a threonyl residue on the peptide. The strength of this signal relative to those of the other anomeric carbons suggest that most or all of the oligosaccharides are attached to threonyl residues, rather than to seryl residues. Allerhand et al. [100] have demonstrated that ManαThr and ManαSer standards generate C_1 signals 1 ppm apart (at 102.8 and 101.8 ppm, respectively), so

FIGURE 14

Proton decoupled ^{13}C-NMR spectra of cellobiohydrolase I(D) and the oligosaccharides released from cellobiohydrolase I(D) by reductive ß-elimination.

Spectra of (A) 400 mg CBH I(D), (B) 10.5 mg reduced trisaccharide from ß-elimination of CBH I(D) and (C) 2.0 mg reduced disaccharide from ß-elimination of CBH I(D), were recorded at 75.46 MHz. Chemical shifts are reported in parts per million from internal TSP. Spectra were the result of (A) 35712 accumulations, (B) 4304 accumulations and (C) 16328 accumulations, respectively. For further details, see "Experimental Procedures."

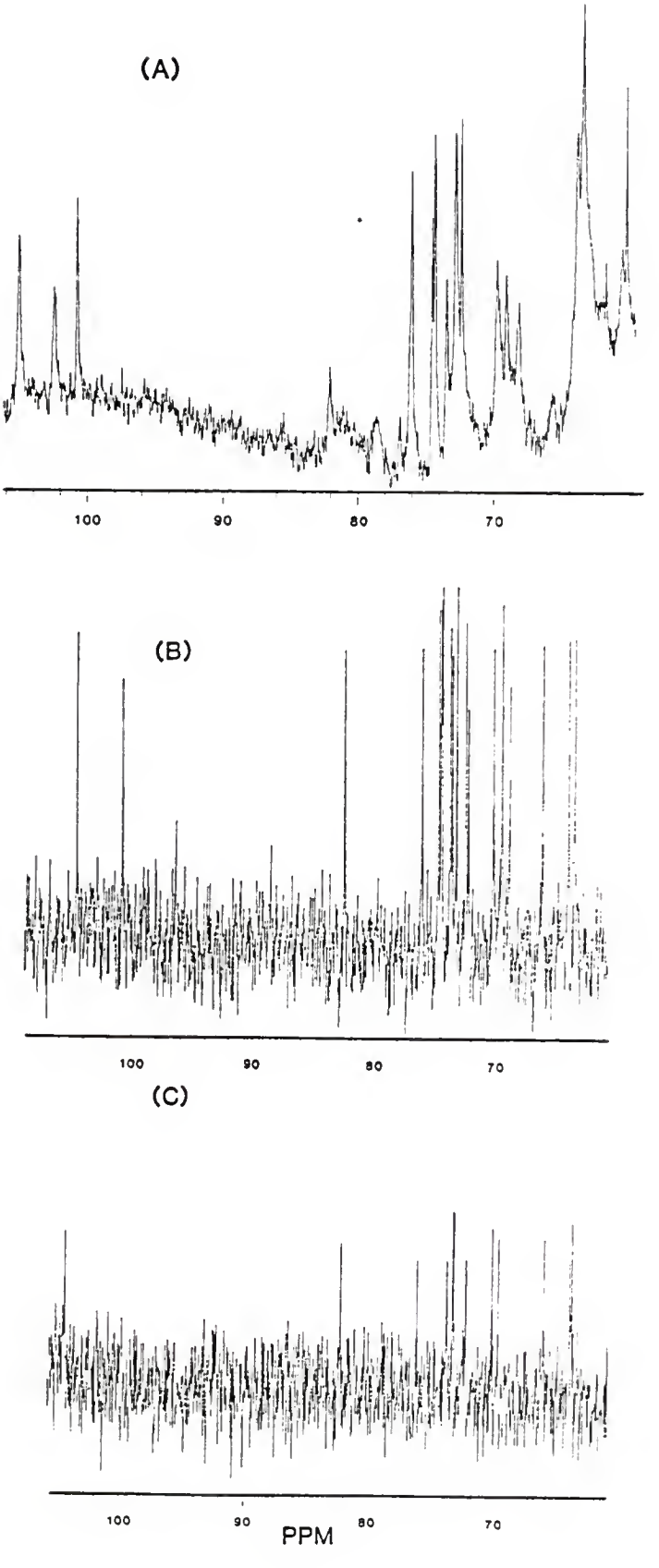

(A)
100
90
80
70
(B)
100
90
80
70
(C)
100
90
80
70
PPM

TABLE V

SELECTED ^{13}C-NMR SIGNALS DUE TO CARBOHYDRATE COVALENTLY ATTACHED TO CELLOBIOHYDROLASE I(D) AND DUE TO PURIFIED OLIGOSACCHARIDES RELEASED FROM THIS ENZYME BY REDUCTIVE β-ELIMINATION

Proton coupled and decoupled ^{13}C-NMR spectra were performed at 75.5 MHz. 200 mg CBH I(D), 10.5 mg reduced trisaccharide and 2.0 mg reduced disaccharide were used. Chemical shifts are expressed in PPM and, in parentheses, $^{1}J_{C,H}$ values are expressed in Hz. For further details, see "Experimental Procedures".

	Chemical Shifts Relative to TSP			
Carbon Atom	CBH I(D)	Reduced Trisaccharide	Reduced Disaccharide	Residue
Anomeric Carbons				
C-1	102.5 (171.1)	-	-	Man
C-1'	105.1 (173.6)	104.6 (169.2)	104.3	Man
C-1"	100.8 (171.5)	100.9 (170.5)	-	Glc
Hydroxymethyl Carbons				
C-1	-	66.0	66.0	Mannitol
C-6	63.9	63.9	64.0	Man
C-6'	-	-	63.8	Man
C-6"	63.4	63.4	-	Glc

attachment to more than one type of hydroxy amino acids would be expected to result in separate signals, which is not the case here. Protein folding is thought not to affect the chemical shift of carbohydrate residues [101], as it does the chemical shift of amino acid residues.

The above data is in agreement with sequence evidence from Pettersson [60] which suggested that all of the O-linked carbohydrate is present in a 20 amino acid region located 33 residues from the C-terminus of CBH I(D); the work of Shoemaker et al. [61], who have sequenced the gene for the enzyme, suggests that the region contains 8 threonyl and 3 seryl residues. This is also in agreement with amino acid analysis of the polypeptide from which the carbohydrate had been removed by ß-elimination (see below).

Comparison of the CBH I(D) anomeric carbon signals with those of the reduced trisaccharide shows the loss of the signal at 102.5 ppm (Fig. 14,Panel B). This was expected as the linking sugar has now been reduced and thus has no anomeric carbon atoms. This further confirms that this signal is due to the mannosyl residue attached to the protein. The signals at 105.1 ppm (C-1') has been shifted to 104.6 ppm, suggesting this mannosyl residue is close to the reducing end. The signal at 100.9 ppm is unchanged, suggesting that this glucosyl residue is furthest removed from the reducing end. This data is in agreement with the chemical evidence of the sequence of sugars in the trisaccharide (see above). Spectra of the reduced disaccharide reveal a single signal at 104.3 ppm (C-1') due to the non-reducing terminal mannosyl residue (Fig. 14,Panel C). Comparison of the signals in the hydroxymethyl region (63-66 ppm), indicates that the carbohydrate of

intact CBH I(D) (Fig. 14,Panel A) generates two signals. Thus the trisaccharide, as it is attached to the protein, has only two hydroxymethyl groups and must contain one 6-substituted residue. The reduced trisaccharide reveals three signals, and thus it must contain one 6-substituted residue (Fig. 14,Panel B). The reduced disaccharide also gives rise to three signals, which suggests that it cannot contain a 6-substituted residue (Fig. 14,Panel C).

Coupled ^{13}C-NMR spectra performed on both CBH I(D) and the reduced trisaccharide, provide coupling constants for anomeric carbons and yield information on the nature of those carbons. All $^{1}J_{CH}$-values (Table V) for anomeric carbons of both CBH I(D) and the reduced trisaccharide were between 169.2-173.6 Hz indicative of α-linked sugar residues (ß-linkages generate J-values of about 160 Hz), in agreement with the results of the glycosidase digestion (see above).

^{1}H-NMR--The ^{1}H-NMR signals of anomeric protons at 300 MHz of the reduced oligosaccharides ß-eliminated from CBH I(D) are shown (Fig. 15). As with the ^{13}C-NMR, the reducing end residues do not contain anomeric carbons and do not generate signals in this region. At this resolution, with a 300 MHz instrument, distinction between signals separated by less than 2 Hz was not possible and thus it was not possible to distinguish between α- and ß-anomers of mannose by these spectra. Signal assignments are shown in Table VI. The glucose signals generated from the tri- and tetrasaccharides are split by 3.5 Hz and 3.4 Hz, respectively, due to the equatorial proton at C_1 and the axial proton at C_2, which indicates that both residues are

FIGURE 15

Proton-NMR spectra at 300 MHz of oligosaccharides released from cellobiohydrolase I(D) by reductive ß-elimination.

Spectra were generated from (A) 2.0 mg reduced disaccharide, (B) 10.5 mg reduced trisaccharide and (C) 700µg reduced tetrasaccharide in D_2O. All spectra were recorded at 25°C and were the result of 200-300 accumulations. Spectra at 70°C revealed no signals "hidden" under the large HOD peak at 4.771 ppm. For further details see "Experimental Procedures".

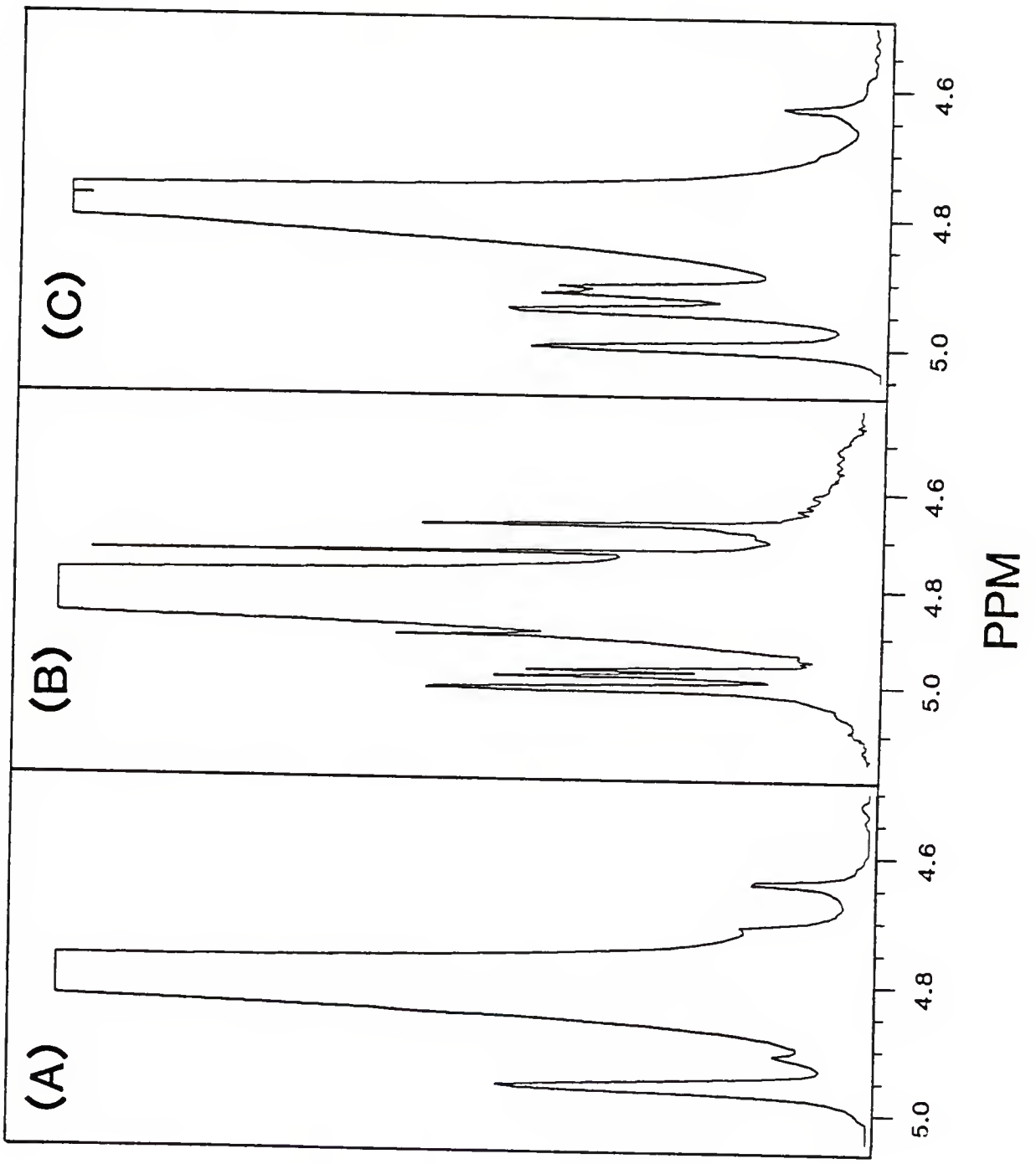

(A)
(B)
(C)
5.0
4.8
4.6
PPM

TABLE VI

^{1}H-NMR SIGNAL ASSIGNMENTS OF ANOMERIC PROTONS OF PURIFIED OLIGOSACCHARIDES RELEASED FROM CELLOBIOHYDROLASE I(D) BY REDUCTIVE β-ELIMINATION

Oligosaccharide	Anomeric Proton	Sugar	Chemical Shift (PPM)	J_{H-H}	Anomer
Disaccharide	H'	Man	4.953	<2.0	α or β
Trisaccharide	H'	Man	4.995	<2.0	α or β
	H"	Glc	4.969/ 4.957	3.5	α
Tetrasaccharide	H'	Man	4.998	<2.0	α or β
	H"	Glc	4.916/ 4.906	3.4	α
	H'"	Man	4.942	<2.0	α or β

present in the α-configuration. The addition of a terminal mannosyl residue on the tetrasaccharide compared to the trisaccharide resulted in an upfield shift of the anomeric glucose signal of 0.05 ppm. The signals at 4.995 ppm and 4.998 ppm were assigned to the mannosyl residues penultimate to the reducing end of the reduced tri- and tetrasaccharides as these residues were in equivalent positions in the respective molecules. Confirmation of these spectral assignments is not possible at this time as these are unique oligosaccharides and standards are not available.

Amino Acid Analysis--α-Amino-butyric acid (Abu) produced from the β-elimination and reduction of CBH I(D) was measured to determine the possible number of oligosaccharide chains attached to threonine on the proteins. A control experiment without β-elimination but in the presence of $PdCl_2$ yielded a background of 4.9 ± 0.2 moles of Abu per mole CBH I(D). The origin of this background Abu is not known. Following β-elimination, 11.8 ± 0.6 moles of Abu per mole CBH I(D) was found. Accounting for the control, there are 6.9 ± 0.7 moles Abu per mole CBH I(D) produced following β-elimination of O-linked oligosaccharides. This value of 6.9 ± 0.7 oligosaccharide side chains agrees with the value for side chains determined from analysis of carbohydrate composition by GLC (5.9 ± 0.6) and HPLC (7.2) analyses. It also is in agreement with evidence from the ^{13}C-NMR and other sequence data [60,61] that most or all of the carbohydrate is attached to threonine.

Assessment of CBH I(D) Oligosaccharide Structure--From the data presented, it is clear that the oligosaccharides covalently attached to CBH I(D) are a related series. The mono-, di-, tri- and tetrasaccharides have been shown to be Man, Manα(1-2)Man, Glcα(1-6)Man α(1-2)Man and Manα(1-2)Glcα(1-6)Manα(1-2)Man, respectively. These have been shown to be attached primarily to threonyl residues on the polypeptide.

Cellobiohydrolase II

Molecular Properties of CBH II--CBH II has previously been shown to have a molecular weight of 54,000 daltons by sedimentation equilibrium and carbohydrate and amino acid analysis and comprises about 20% of the extracellular protein secreted by Trichoderma reesei QM 9414 grown on cellulose [49]. The material eluting at pH 6.0 from the DEAE-Sephadex column (fractions 45-105, Fig.1) and which contained the enzyme, was pooled and applied to an SP-Sephadex column. Isocratic elution of the SP-Sephadex with 8mM sodium succinate with 3mM sodium azide at pH 4.5, resulted in CBH II which was pure by the criterion of polyacrylamide disc gel electrophoresis (Figs. 2 and 16). This protein was also shown to yield a single band by horizontal isoelectric focusing, and was determined to be isoelectric at pH 5.91 ± 0.02, which was slightly higher than that of pH 5.6 found earlier [49]. Immunodiffusion using antisera produced against CBH II yielded a single precipitin band with the homologous protein, but no cross-reaction with CBH I(D), or any endoglucanases tested. An experimentally determined extinction coefficient of 15.62 ± 0.19

FIGURE 16

Polyacrylamide disc gel electrophoresis of crude extracellular protein prepared from T. reesei QM 9414 and highly purified cellobiohydrolase II.

Lanes 1 and 2 contain 100 µg each of crude protein. Lanes 3 and 4 represent 30 µg each of highly purified CBH II (from pool of fractions 110-150) eluted from SP-Sephadex with 8 mM sodium succinate, 3 mM sodium azide, pH 4.5 (see Fig. 2). Lanes 1 and 3 were stained with Coomassie Brilliant Blue for protein and lanes 2 and 4 were stained with the periodic acid-Schiff reagent for carbohydrate.


1
2
3
4

(for a 1% solution at 280 nm) to estimate CBH II concentrations was used throughout the experiments reported here. Pure CBH II was found to have an A_{280}/A_{260} ratio of 1.82-1.84. The extinction coefficient for this protein was determined in 50mM phosphate buffer at pH 7.0, because after extensive dialysis against water, lyophillization and drying over phosphorous pentoxide, CBH II did not dissolve completely, unless counterions were present. This requirement for counterions to ensure complete solubility was apparent during later NMR experiments in which CBH II precipitated during analysis of a solution in D_2O.

Neutral Carbohydrate Composition--As with CBH I(D), the carbohydrate was removed from CBH II either by reductive ß-elimination to release O-linked oligosaccharides or by mild acid hydrolysis to release all sugars as monosaccharides. The products were then converted to the respective peracetylated alditols and aldononitriles and analyzed by gas-liquid chromatography (Table VII). The protein was found to contain about 42 mannose and 13 glucosyl residues which together accounted for 18.2 percent by weight of the glycoprotein. This was less than the 51 mannose and 20 glucose residues found earlier [49], although protein was estimated by the Lowry method in that case, and not by extinction coefficient. As mentioned previously analysis of the derivatized products released by reductive ß-elimination provided information as to the total carbohydrate composition, the number of O-linked attachment sites and the type of sugar involved in the attachment to the protein (Table VII). So for CBH II, mannose (identified as the peracetylated

TABLE VII

NEUTRAL CARBOHYDRATE COMPOSITION OF CELLOBIOHYDROLASE II

CBH II was treated with either mild acid or alkaline borohydride and the products analyzed either as a mixture of PAANs and PAAs or, after reduction with sodium borohydride, as the PAAs. For further details, see "Experimental Procedures." Values are expressed in moles/mole protein.

Peracetylated Alditols and Aldononitriles	Method of Carbohydrate Release	
	Acid Hydrolysis	Alkaline Borohydride
Mannononitrile	42.6 ± 1.4	16.2 ± 1.4
Glucononitrile	13.1 ± 0.6	11.8 ± 1.5
Mannitol		24.7 ± 1.9
Mannitol	42.2 ± 2.0	42.3 ± 1.5
Glucitol	13.1 ± 1.1	12.5 ± 0.9

mannitol) was the only saccharide found to be O-linked to the protein and was estimated to form an average of 25 sites of attachment per molecule glycoprotein. The fact that the total number of neutral sugars recovered from either reductive ß-elimination or acid hydrolysis were the same suggested that all the neutral carbohydrate was alkali-labile (i.e. O-linked). CBH II then, contains more covalently attached carbohydrate enzyme molecule than does CBH I(D), but it also possesses larger number of oligosaccharide side chains, 25 in comparison to six or seven found in CBH I(D).

O-linked Oligosaccharides--O-linked oligosaccharides released from CBH II by reductive ß-elimination were separated by gel filtration on Biogel P-2 (see Fig. 17), purified by rechromatography on the same column and weighed (see Table VIII). Analysis of the eluate showed an average of 14.9 chains of the trisaccharide, 1.6 chains of the disaccharide and 9.0 chains of the monosaccharide. This accounted for about 25.5 oligosaccharide chains O-linked to CBH II in good agreement with the gas chromatography results (see above). The 18.1% total neutral carbohydrate calculated also agrees well with previous evidence from gas chromatography.

Analysis of the composition of each of the oligosaccharides by gas-liquid chromatography (see Table IX) provided evidence that, like those from CBH I(D), these may form a related series. All were found to contain mannose (determined as the peracetylated mannitol) at the reducing terminus, and the trisaccharide also was found to contain a glucosyl residue. The nearly integral values observed for each component residue by compositional analysis (GLC) and the symmetry of peak shapes from HPLC of the oligosaccharides after ß-elimination

FIGURE 17

Separation on a Biogel P-2 column of oligosaccharides released from cellobiohydrolase II by reductive ß-elimination.

50 mg CBH II was ß-eliminated with 0.1M NaOH and 0.3 M $NaBH_4$ at 40°C for 48 hours. The reaction was stopped with 2N acetic acid and the mixture taken to dryness. Following methanol evaporation, the reduced oligosaccharides were deionized over Amberlite MB-3, lyophillized and then applied to a Biogel P-2 column. For further details see "Experimental Procedures". Peaks labelled M, M_2 and M_2G correspond to mono-, di- and trisaccharides, respectively.

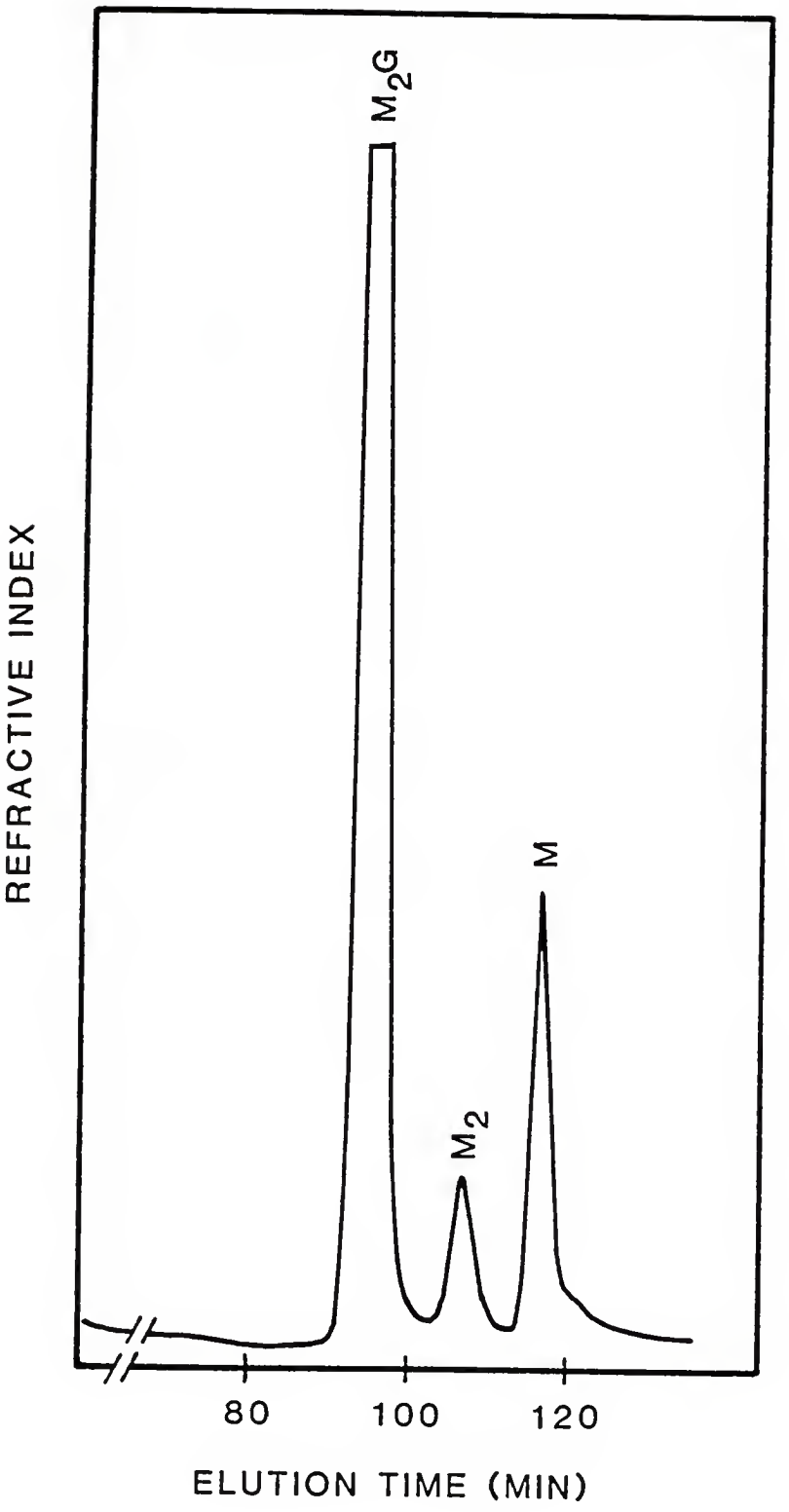

REFRACTIVE INDEX
M_2G
M
M_2
80
100
120
ELUTION TIME (MIN)

TABLE VIII

OLIGOSACCHARIDES RELEASED BY PREPARATIVE
ß-ELIMINATION OF CELLOBIOHYDROLASE II

Oligosaccharides released by reductive ß-elimination of CBH II were separated on Biogel P-2 (see Fig. 17), pooled and lyophillized. Samples were then dried over P_2O_5 and weighed. Calculations were made assuming a molecular weight for CBH II of 54,000 daltons [49].

Oligosaccharide	Weight Percent of Carbohydrate	Weight Percent of Glycoprotein	Moles per Mole CBH II
Tri-	76.5	14.0	14.9
Di-	5.3	1.0	1.6
Mono-	18.2	3.1	9.0
		18.1%	25.5

TABLE IX

COMPOSITION OF OLIGOSACCHARIDES RELEASED FROM CELLOBIOHYDROLASE II BY REDUCTIVE ß-ELIMINATION

Reduced oligosaccharides, purified on Biogel P-2, were hydrolyzed with mild acid and the products analyzed by gas-liquid chromatography as a mixture of the alditol and aldononitrile acetates. For further details, see "Experimental Procedures".

Peracetylated Derivative	Saccharide		
	Mono-	Di-	Tri-
Mannitol[a]	1.0	1.0	1.0
Mannononitrile	-	0.9	1.2
Gluconononitrile	-	-	0.9

[a] mannitol hexaacetate has been normalized to 1.0 assuming one reducing end per oligosaccharide chain prior to ß-elimination.

suggest a homologous and related series of side chains, as was found for CBH I(D).

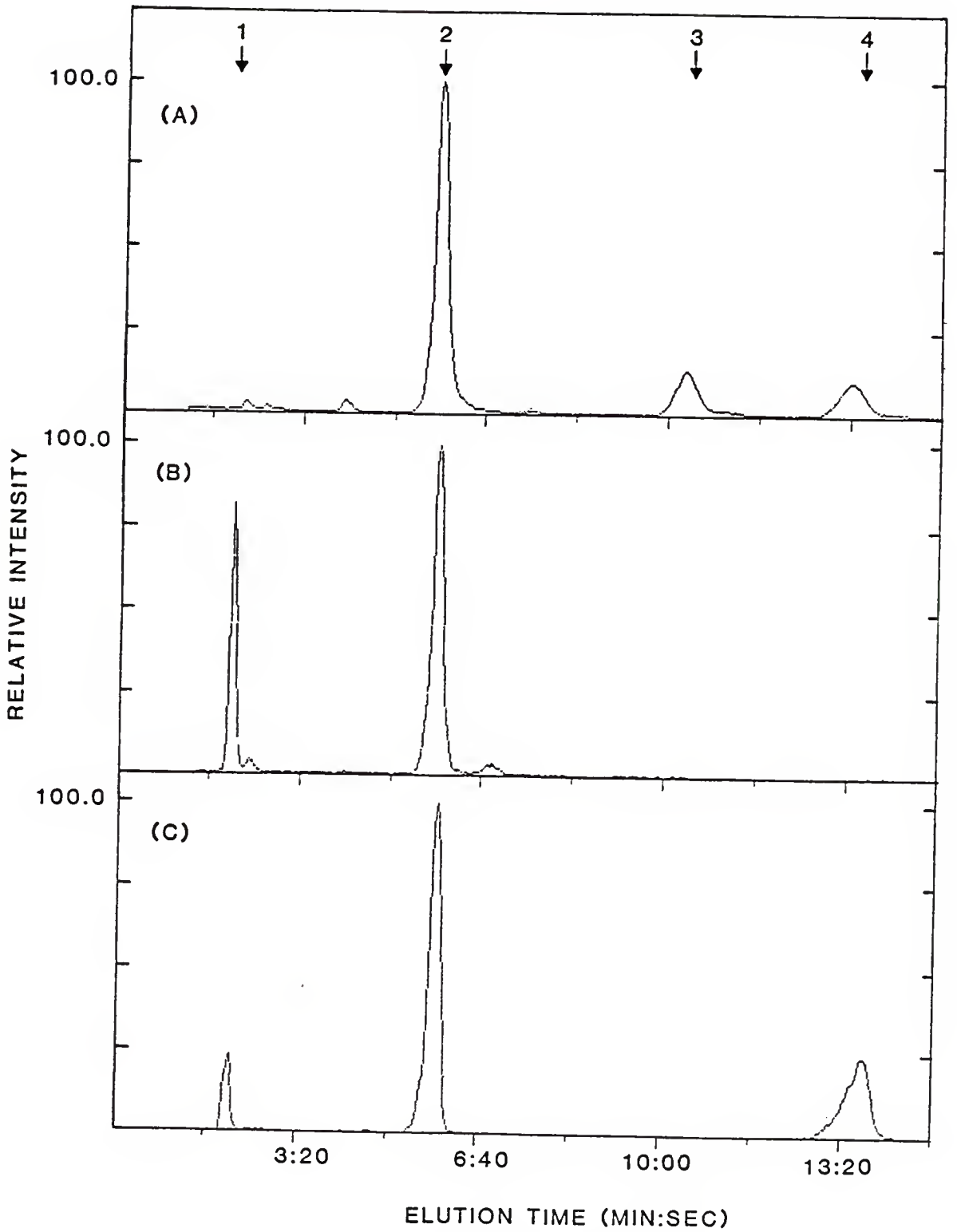
Methylation Analysis--The types of linkages between the sugar residues were studied by methylation analysis. Partially methylated peracetylated alditols from the intact CBH I(D) and the oligosaccharides released from the glycoprotein by reductive ß-elimination were separated and identified by gas chromatography/mass spectrometry (Fig. 18) and the electron-impact spectra were compared with those of prepared from standard carbohydrates (examples are shown in Fig. 11 and in Appendix C). It should be mentioned again that glucitol and mannitol sustituted identically with methyl and acetyl groups were indistinguishable as they were eluted together on the gas chromatograph and also were fragmented identically by electron impact.

Intact CBH II yielded three methylated species corresponding to the 2,3,4,6-tetramethyl, 3,4,6-trimethyl and 2,3,4-trimethyl alditol acetates indicating the presence of non-reducing terminal 2-substituted and 6 substituted hexoses, respectively (Fig. 18,Panel A). No dimethyl hexitol acetates were observed indicating that all oligosaccharide chains were unbranched. The ratio of the 3,4,6-/2,3,4-species was 0.95, which agrees well with the estimated ratio (0.90) calculated from the relative abundance of tri- and disaccharide attached to CBH II (Table VIII). The reduced disaccharide formed during ß-elimination produced two partially methylated alditol acetates corresponding to the 1,3,4,5,6-pentamethyl and 2,3,4,6-tetramethyl species indicating a (1-2) linked mannobiitol (Fig. 18,Panel B). Analysis of the reduced trisaccharide also revealed

FIGURE 18

Methylation analyses of cellobiohydrolase II and the oligosaccharides released from cellobiohydrolase II by reductive ß-elimination.

G.C./M.S. profiles of partially methylated alditol acetates generated from (A) CBH II (B) reduced disaccharide and (C) reduced trisaccharide. Individual peaks were positively identified by their electron-impact spectra compared with prepared standards for further details, see "Experimental Procedures". Arrows indicate elution of standards: 1. 2-O-acetyl-1,3,4,5,6-penta-O-methyl glucitol; 2. 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl mannitol; 3. 1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl mannitol; 4. 1,5,6-tri-O-acetyl- 2,3,4-tri-O-methyl mannitol.


1
2
3
4
100.0
(A)
100.0
(B)
100.0
(C)
RELATIVE INTENSITY
3:20
6:40
10:00
13:20
ELUTION TIME (MIN:SEC)

three peaks corresponding to the 1,3,4,5,6-pentamethyl, 2,3,4,6-tetramethyl and 2,3,4-trimethyl hexitol acetates (Fig. 18,Panel C). This indicates the presence of 2-substituted (reduced end), non-reducing end and 6-substituted residues, respectively. As with the reduced oligosaccharides from CBH I(D), no dimethyl hexitol acetate species were found, indicating the absence of branched chains.

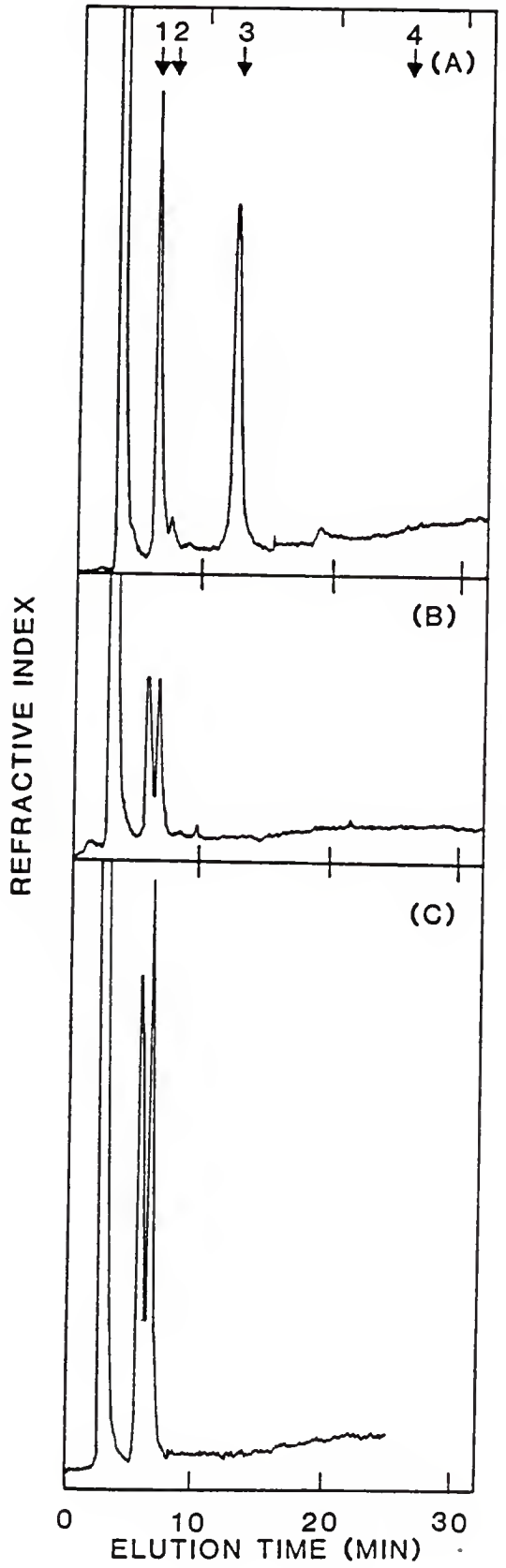
Since the monosaccharides attached to the protein should yield exclusively tetramethyl alditol acetates and since there is very little disaccharide, the pattern of the trimethyl species generated for the intact CBH II (Fig. 18,Panel A) should correspond closely to that expected if the trisaccharide were the only side chain attached to the protein. Comparison of the elution of partially methylated alditol acetates from this glycoprotein (Fig. 18,Panel A) with that from the reduced trisaccharide (Fig. 18, Panel C) obtained from CBH II, reveals the absence of one trimethyl species in the latter; a new pentamethyl species from the reduced end of the trisaccharide was now present. The missing peak was identified as the 3,4,6-trimethyl species, indicating that the residue attached to the protein is substituted in the 2-position. This was supported by the identification of the 1,3,4,5,6-pentamethyl hexitol acetate in the trisaccharide, which confirmed the position of the 2-substituted sugar at the reducing end of the side chain. The methylation profiles, then, in conjunction with the evidence from glycosidase digestion (see below), suggest two related oligosaccharides, Glc(1-6)Man(1-2)mannitol and Man(1-2)mannitol, attached to CBH II.

Glycosidase Digestions--Oligosaccharides released from CBH II by reductive ß-elimination were subjected to sequential glycosidase

FIGURE 19

HPLC separation of the products of sequential glycosidase digestion of oligosaccharides released from cellobiohydrolase II by reductive ß-elimination.

Shown are products from (A) CBH II trisaccharide + α-glucosidase, (B) disaccharide from (A) + α-mannosidase and (C) CBH II disaccharide + α-mannosidase. Elution times of standards are shown: 1. mannose (glucose); 2. mannitol; 3. CBH I(D) reduced disaccharide; 4. CBH I(D) reduced trisaccharide. Separations were performed on a Whatman Partisil PXS 10/25 PAC reverse phase column eluted with 77% acetonitrile at a flow rate of 1.5 ml/min.


1 2
3
4
(A)
(B)
(C)
REFRACTIVE INDEX
0
10
20
30
ELUTION TIME (MIN)

digestion to determine the sequence of sugar residues and the anomeric nature of the glycosidic bonds (Fig. 19). The products of digestion were separated by polar reverse phase HPLC. The monosaccharides released were converted to the peracetylated alditols and/or aldononitriles and identified by gas-liquid chromatography; the resultant shortened oligosaccharides then were degraded with an alternate glycosidase.

The monosaccharide released from CBH II reduced trisaccharide with α-glucosidase (Fig. 19,Panel A) was identified as the peracetylated gluconononitrile (Fig. 19, Panel A) by gas-liquid chromatography. The reduced trisaccharide was not a good substrate for the α-glucosidase and so large amounts (four units) of enzyme were necessary for complete digestion. Consequently, small amounts of mannose and mannitol were produced by a minor α-mannosidase contamination present in this preparation. All non-reducing terminal mannose residues, however, from the disaccharide produced in Panel A (Fig. 19,Panel B) and from the disaccharide released from CBH II by reductive ß-elimination (Fig. 19,Panel C), were readily cleaved by α-mannosidase. Recovery of the products after the digestion and chromatography step was about 85% in each cases.

From the HPLC profiles glycosidase hydrolysates, it is evident that each of the anomeric carbons in glycosidic linkages is in the α-configuration. Thus the sequence of the tri- and disaccharides obtained by reductive ß-elimination of CBH II is proposed to be GlcαManαmannitol and Manαmannitol, respectively.

Acetolysis--The reduced trisaccharide from CBH II was subjected to acetolysis in order to confirm the position of the (1-6)

glycosidic linkage within the oligosaccharide chain. Under carefully controlled conditions, (1-6) glycosidic bonds have been shown to be more susceptible to acetolysis than are the (1-2), (1-3) and (1-4) linkages [71].

The products of the acetolysis reaction were separated by HPLC (Fig. 20) and after hydrolysis further analyzed as the peracetylated alditols and/or aldononitriles by gas-liquid chromatography. The trisaccharide was cleaved into two products, a monosaccharide, which coeluted with a glucose standard and a reduced disaccharide, which coeluted with authentic reduced disaccharide from CBH I(D). The fact that the two disaccharides elute at the same place by polar reverse hase HPLC suggest that they are identical since a variety of standard disaccharides were found to be eluted at different times. Derivatization of the products and identification by gas chromatography demonstrated that the monosaccharide had been converted to the peracetylated gluconononitrile and that the disaccharide yielded a 1:1 mixture of peracetylated mannitol and peracetylated mannononitrile. This was consistent with a terminal glucosyl residue linked by a (1-6) glycosidic bond to mannobiitol.

^{13}C-NMR--Proton decoupled ^{13}C-NMR were obtained at 75.5 MHz for CBH II and for the trisaccharide released from CBH II with alkaline borohydride treatment (Fig. 21 and Table X). CBH II yielded two major signals in the anomeric region (C_1) at 105.0 and 100.7 ppm; this indicated the presence of two predominant types of hexoses (Fig. 21,Panel A). The signal at 100.7 ppm has been assigned to a glucosyl residue and the signal at 105.0 ppm has been assigned to the central mannosyl residue of the trisaccharide. As with CBH I(D), the

FIGURE 20

HPLC separation of the products of acetolysis of the trisaccharide released from cellobiohydrolase II by reductive ß-elimination.

The oligosaccharide was subjected to acetolysis as described under "Experimental Procedures". 1 and 2 refer to the elution times of glucose and reduced disaccharide from CBH I(D), respectively. Separations were performed on a Whatman Partisil PXS 10/25 PAC column eluted with 75% acetonitrile at a flow rate of 1.5 ml/min.

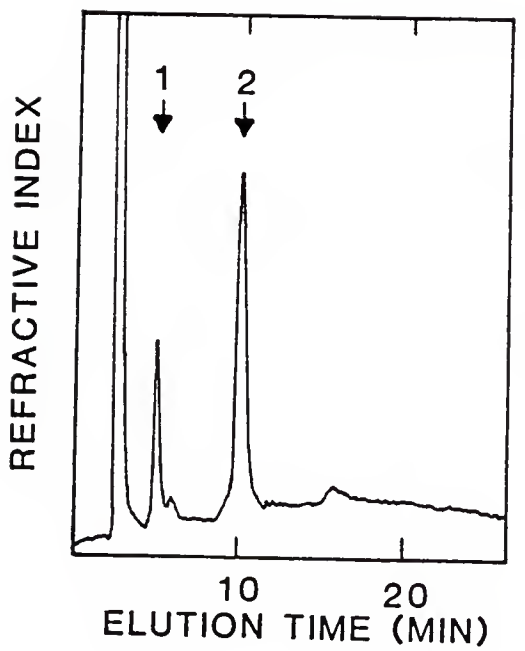
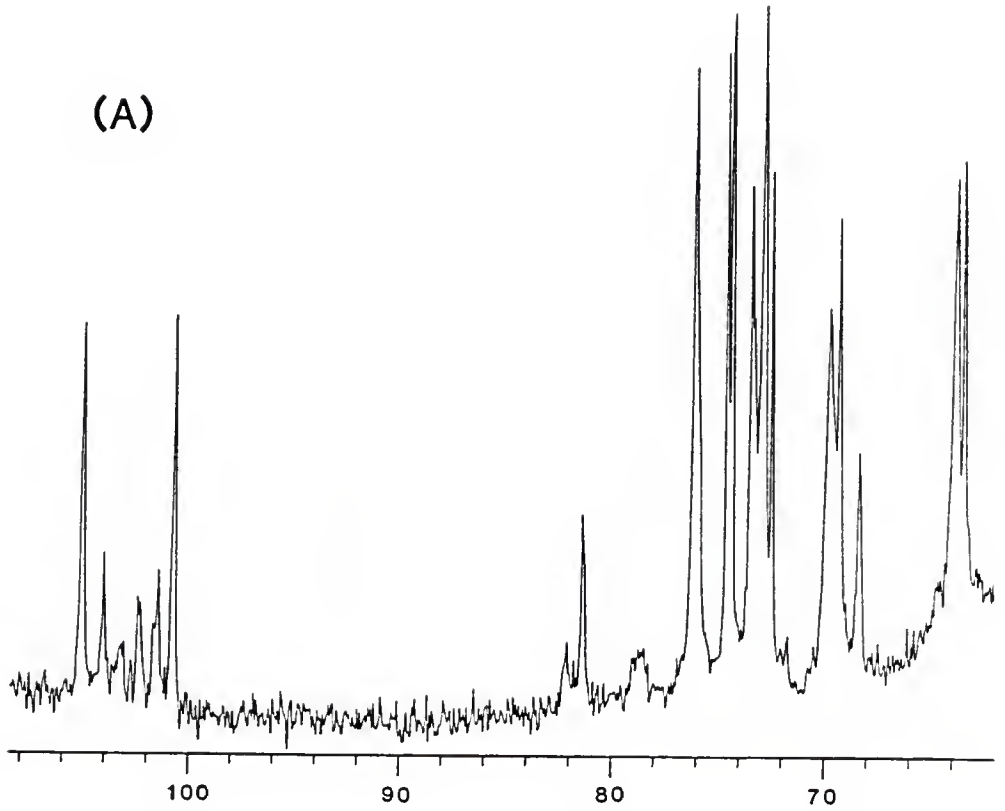

REFRACTIVE INDEX
1
2
10
20
ELUTION TIME (MIN)

FIGURE 21

Proton decoupled ^{13}C-NMR spectra of cellobiohydrolase II and the trisaccharide released from cellobiohydrolase II by reductive ß-elimination.

Spectra of (A) 200 mg CBH II and (B) 5.0 mg reduced trisaccharide from ß-elimination of CBH II, were recorded at 75.46 MHz. Chemical shifts are reported in parts per million from internal TSP. Spectra were the result of (A) 73968 accumulations and (B) 14982 accumulations, respectively. For further details, see "Experimental Procedures."


(A)
100
90
80
70

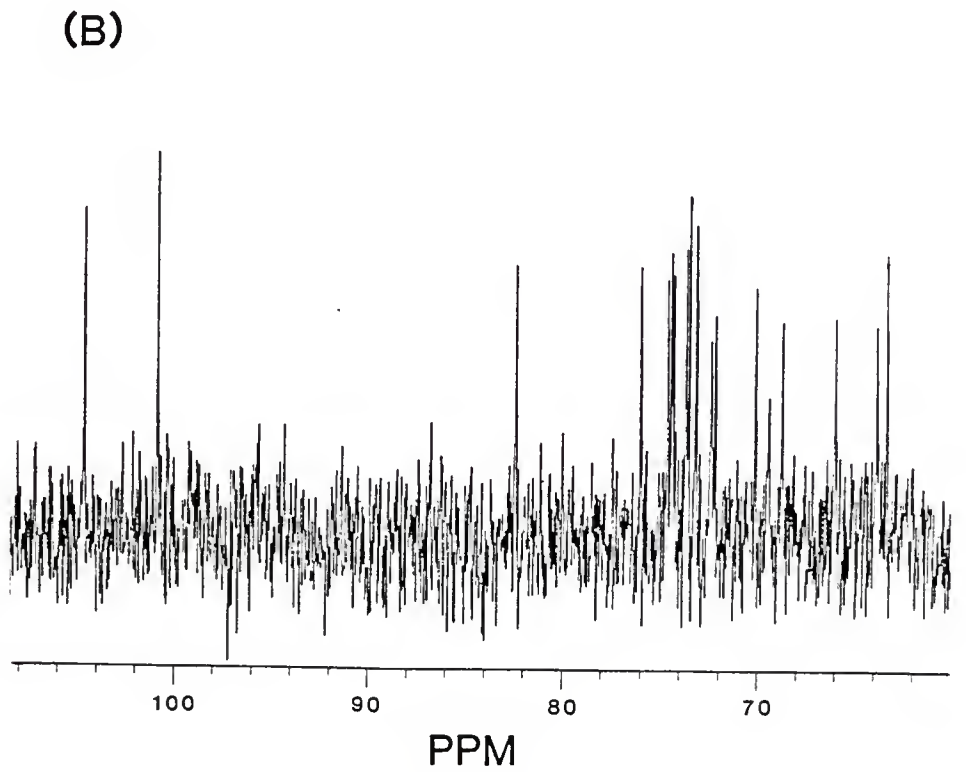

(B)
100
90
80
70
PPM

TABLE X

SELECTED ^{13}C-NMR SIGNALS DUE TO CARBOHYDRATE COVALENTLY ATTACHED CELLOBIOHYDROLASE II AND DUE TO THE PURIFIED TRISACCHARIDE RELEASED FROM THIS ENZYME BY REDUCTIVE β-ELIMINATION

Proton coupled and decoupled ^{13}C-NMR spectra were performed at 75.5 MHz. 200 mg CBH II and 8.0 mg reduced trisaccharide were used. Chemical shifts are expressed in PPM and, in parentheses, $^{1}J_{C,H}$ values are expressed in Hz. For further details, see "Experimental Procedures".

Carbon Atom	Chemical Shifts Relative to TSP: CBH II	Chemical Shifts Relative to TSP: Reduced Trisaccharide	Residue
Anomeric Carbons			
C-1	104.0	-	-
	102.3	-	Man
	101.5	-	Man
C-1'	105.0 (171.8)	104.5	Man
C-1"	100.7 (172.7)	100.8	Glc
Hydroxymethyl Carbons			
C-1	-	66.0	Man
C-6	63.8	63.8	Man
C-6"	63.5	63.4	Glc

latter signal is down field from those of available standards and it is suggested that this is due to the unique nature of the substitution of this residue. Unlike CBH I(D), there is no single predominant anomeric signal for a residue attached to the protein. Instead, there are several small signals in the region, the most predominant being at 104.1, 102.3 and 101.5 ppm. The strength of these signals relative to those of the other anomeric carbons and the fact that neither predominates, suggests that the oligosaccharides are not attached preferentially to any one type of amino acid (ie. threonine or serine). Considering that Allerhand et al. [100] have demonstrated that ManαThr and ManαSer standards generate C_1 signals 1 ppm apart at 102.8 and 101.8 ppm, respectively, one would expect attachment to different amino acids to generate separate signals, which is probably the case here.

From this evidence, and that found for CBH I(D), it was reasonable to assign the signal at 102.3 ppm to a mannosyl residue α-linked to threonine and the signal at 101.5 ppm to a mannosyl residue α-linked to serine on the polypeptide. The signal at 104.0, however, has not yet been assigned as there is no appropriate standards available for comparison. It is possible that this signal is due to a mannosyl residue attached to an amino acid other than serine or threonine, although amino acid analysis of CBH II showed no evidence of hydroxyproline or hydroxylysine. Other possibilities are (i) the presence of relatively small amounts of other species ie. tetrasaccharides attached to CBH II, (ii) some microheterogeneity in the trisaccharide itself, (iii) oligosaccharides ß-linked to the protein or (iv) single mannosyl residues (rather than an

oligosaccharide) attached to an amino acid. The first possibility is unlikely as no larger species were found upon Biogel P-2 separation of all the oligosaccharides released from CBH II by ß-elimination. The residue must be a mannosyl or a glucosyl residue as these are the only neutral sugars attached to the protein as determined by gas chromatographic analysis. That the trisaccharide is heterogeneous is also unlikely as shown by the various chemical analyses performed. A ß-linkage to the protein is a possibility although O-linked oligosaccharides are thought to be linked to the protein in the α-configuration. Unfortunately, the poor resolution of coupled ^{13}C-NMR could not discern this signal. The final possibility is that the signal is due to single mannosyl residues attached to threonine on the protein but it is not known what sort of shift this would cause in the signal. It is known for CBH I(D) that for the trisaccharide, the mannose attached to the protein generates a signal at 102.5 ppm and it has been reported [100] that ManαThr standards generate an anomeric signal at 102.8 ppm. Thus seems unlikely that such a shift would be large enough to account for the difference. Unlike the situation for CBH I(D), there is no sequence data available for CBH II and it is not known to which region(s) of the molecule the carbohydrate side chains are attached. The attachment of oligosaccharide side chains attached to more than one type of amino acid is consistent with the amino acid analysis data presented below.

Comparison of the signals due to anomeric carbons of oligosaccharides of CBH II with those of the reduced trisaccharide (Table X) show a shift in the signal at 105.0 ppm for the former, to 104.5 ppm, in the latter, suggesting that the mannosyl residue was

close to the reducing end and was affected by the ring opening. As expected in the spectrum of the reduced trisaccharide, no signals were found in the linking sugar region between 101 and 104 ppm, as these residues are now reduced and so are no longer anomeric. The signal at 100.8 ppm was unchanged, suggesting that this glucosyl residue was furthest from the reducing end. This data supports chemical evidence of the sequence of sugars in the trisaccharide found by glycosidase digestion and by compositional analysis. Unfortunately, there was not enough of the disaccharide from CBH II to permit a ^{13}C-NMR analysis.

Inspection of the signals in the hydroxymethyl region (63-66 ppm), reveals that intact CBH II (Fig. 21,Panel A) generated two signals suggesting that the trisaccharide attached to the protein has two hydroxymethyl groups and thus must contain one 6-substituted residue. However, the reduced trisaccharide from CBH II (Fig. 21, Panel B) produced three signals (two from the reducing end residue) and so must contain one 6-substituted residue. Apart from the missing signal at 102.5 ppm, the carbohydrate region of the ^{13}C-NMR spectra for CBH II is essentially the same as that of CBH I(D), as are the spectra of the trisaccharides from the two enzymes, indicating that the oligosaccharides are the same.

^{1}H-NMR--The ^{1}H-NMR spectra of anomeric protons at 300 MHz of the reduced oligosaccharides β-eliminated from CBH II were obtained (Fig. 22) and signal assignments are shown in Table XI. As with the ^{13}C-NMR, the reducing end residues do not contain anomeric carbons and so were not expected to generate signals in this region. Under these conditions, it was not possible to resolve signals

FIGURE 22

Proton-NMR spectra at 300 MHz of oligosaccharides released from cellobiohydrolase II by reductive β-elimination.

Spectra were generated from (A) 2.0 mg reduced disaccharide and (B) 5.0 mg reduced trisaccharide in D_2O. All spectra were recorded at 25°C and were the result of 16-200 accumulations. Spectra at 70°C revealed no signals "hidden" under the large HOD peak at 4.771 ppm. For further details, see "Experimental Procedures".

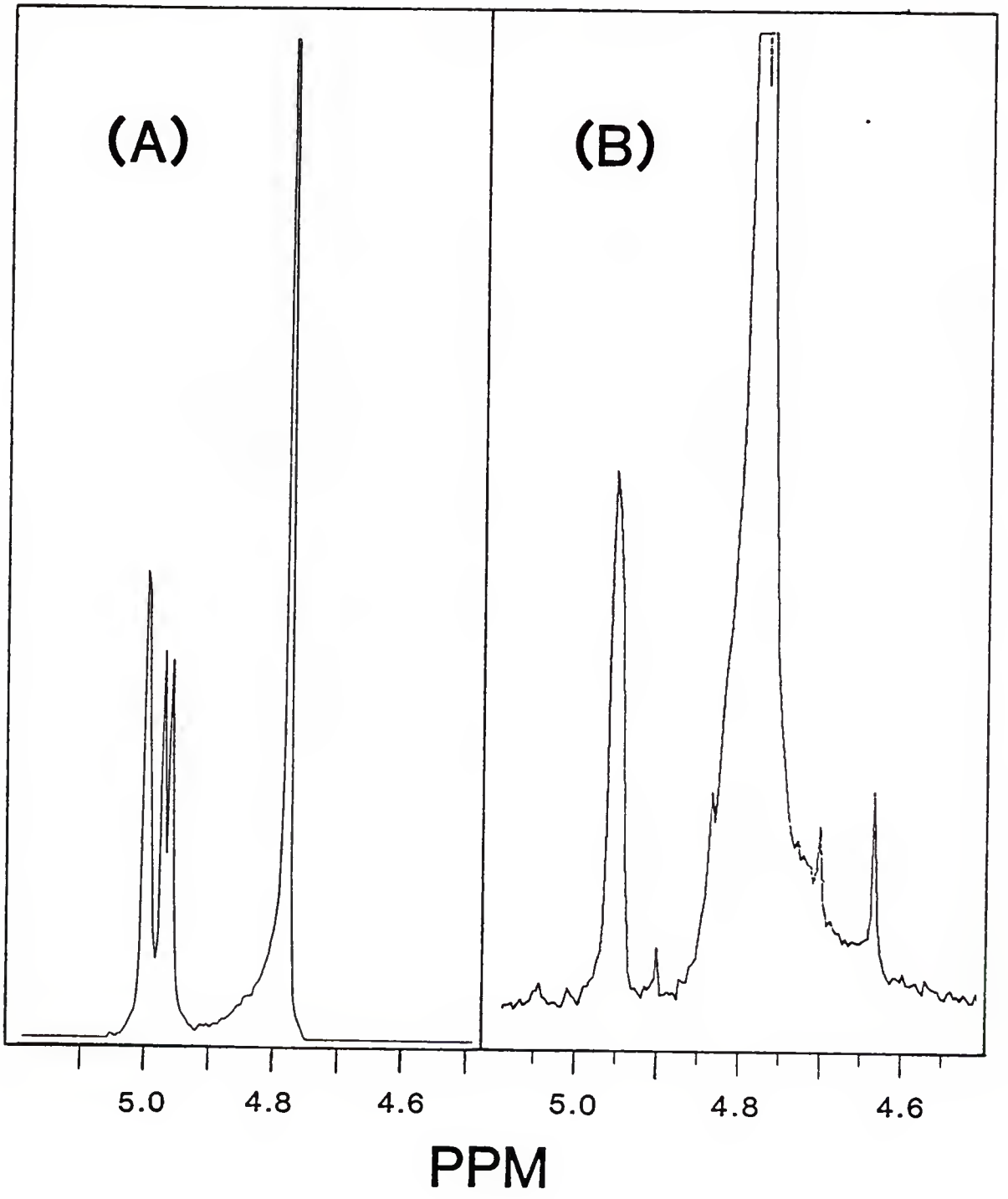

(A)
(B)
5.0
4.8
4.6
5.0
4.8
4.6
PPM

TABLE XI

^{1}H-NMR SIGNAL ASSIGNMENTS OF ANOMERIC PROTONS OF PURIFIED OLIGOSACCHARIDES RELEASED FROM CELLOBIOHYDROLASE II BY REDUCTIVE β-ELIMINATION

Oligosaccharide	Anomeric Proton	Sugar	Chemical Shift (PPM)	J_{H-H}	Anomer
Disaccharide	H'	Man	4.952	<2.0	α or β
Trisaccharide	H'	Man	4.996	<2.0	α or β
	H"	Glc	4.971/ 4.959	3.5	α

separated by less than 2 Hz and thus it was not possible to distinguish between α- and ß-anomers of mannose in these spectra.

The reduced trisaccharide from CBH II gave rise to two signals, one at 4.996 ppm due to a mannosyl residue, and one split signal at 4.959 and 4.971 ppm due to a glucosyl residue. The glucosyl signals generated from this oligosaccharide are split by 3.5 Hz, characteristic of an equatorial proton at C_1 (C_1eq) and an adjacent axial proton at C_2 (C_2ax), which indicated that the anomeric carbons of this residue was present in the α-configuration. As a point of reference, a glucosyl residue with a ß-configuration, which has axial protons at C_1 and C_2, would be expected to have a splitting of 8.0 Hz, easily identified at this resolution. The disaccharide from CBH II gave rise to a single unsplit signal in the anomeric region at 4.952 ppm, indicative of a mannosyl residue. The ^{1}H-NMR spectra generated from the reduced oligosaccharides from CBH II are essentially identical with those from CBH I(D), indicating that each corresponding member of these two series has the same composition and structure. The NMR data is consistent with previous chemical evidence.

Amino Acid Analysis--α-Amino-butyric acid (Abu) produced from the ß-elimination and reduction of CBH II was measured to estimate the number of oligosaccharide side chains attached to threonine on the proteins. A control experiment without ß-elimination but in the presence of $PdCl_2$ yielded a background of 2.2 ± 0.1 moles of Abu per mole CBH II, although the origin of this background Abu is unknown. After ß-elimination, 13.0 ± 0.3 moles of Abu per mole CBH II was found. Therefore, accounting for the control level, there were

10.9 ± 0.4 moles Abu per mole CBH II produced by ß-elimination of O-linked oligosaccharides.

A value of 11 side chains is less than half of the total of 25 linkage sites determined by gas-liquid chromatography (Table VII) and the 26 oligosaccharides estimated by HPLC (Table VIII). This suggests that less than half of the oligosaccharides are attached to threonine residues and is consistent with the ^{13}C-NMR evidence for CBH II, which implies that the side chains are not attached predominantly to one type of amino acid.

Assessment of CBH II oligosaccharide structure--These data suggest that the oligosaccharides attached to CBH II form a related series. The mono-, di- and trisaccharides have been shown to be Man, Manα(1-2)Man and Glcα(1-6)Manα(1-2)Man, respectively. The configuration of the anomeric carbon atom glycosidically linking the oligosaccharides to the polypeptide has not been established, although it is probably in the α-configuration. The side chains were shown to be attached at 25 or 26 sites to both serine and threonine residues on the protein and may also be attached to some other, as yet unidentified, amino acid.

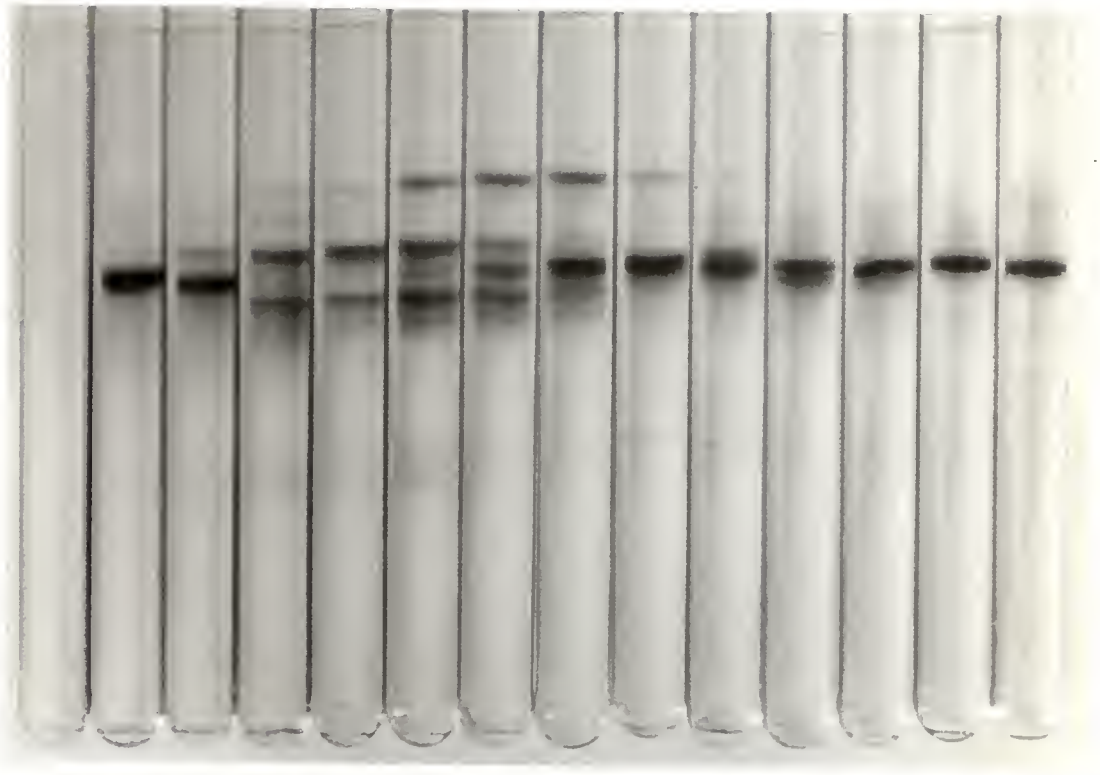
Endoglucanases

Purification of Endoglucanases--All of the endoglucanase activity from an extracellular preparation of Trichoderma reesei QM 9414 grown on cellulose, was eluted isocratically at pH 6.0 from a DEAE-Sephadex column (Fig. 1). This endoglucanase activity also was

FIGURE 23.

Polyacrylamide disc gel electrophoresis of successive protein fractions eluted isocratically from an SP-Sephadex column during chromatography of *T. reesei* endoglucanases.

Shown are gels of fractions 10-37 monitoring the separation of the endoglucanases on SP-Sephadex (see Fig. 2). All gels were stained for protein with Coomassie Brilliant Blue. 40 µg of protein was applied to each gel and gel C was a control with no protein added. For further details, see "Experimental Procedures".


C
10
12
14
16
18
20
22
24
26
28
31
34
37

eluted isocratically with 2mM sodium succinate containing 3mM sodium azide at pH 4.5, from an SP-Sephadex column (Fig. 2). The proteins were eluted from this column at pH 4.5 in order to separate endoglucanase E (Endo E), which was retarded under these conditions (Fig.23). Elution at pH 5.0, the method which was performed previously [49], did not achieve this separation. The endoglucanases were named Endo B, Endo D and Endo E due to the order in which protein peaks emerged from an SP-Sephadex column during enzyme purification. These names are provisional only and will be modified as additional information regarding structure and activity become available.

After unsuccessfully attempting to further purify Endo E using a pH gradient, a pool of this protein from the SP-Sephadex column (fractions 22-40) was applied to a Sephacryl S-200 column (2.5 x 95 cm) equilibrated with 20mM sodium succinate with 3mM sodium azide at pH 5.0. Endo E was eluted in high purity (Fig. 24) at a flow rate of 15 ml/h and was separated from a major contaminant protein, as determined by polyacrylamide disc gel electrophoresis (Fig. 25). Purified Endo E was determined to have a specific activity of 184.7 $\Delta\emptyset_{sp}$/min/mg protein on carboxymethylcellulose and 8.6 µmoles glucose released/min/mg protein on Walseth cellulose.

Endoglucanases B and D (Endo B and Endo D) were separated from one another with the use of a pH gradient as it was found that both were bound to SP-Sephadex at pH 4.0 and low ionic strength. The pool of material containing both Endo B and Endo D (fractions 13-21) from the first SP-Sephadex column (Figs. 2 and 23) was applied to an SP-Sephadex column (4.4 x 55 cm) equilibrated with 2mM sodium succinate with 3mM sodium azide at pH 4.0. Endo B and Endo D were

FIGURE 24

Elution pattern from Sephacryl S-200 column chromatography of endoglucanase E.

Conditions of elution are described in "Results and Discussion: Endoglucanases". The volume of each fraction was 5.25 ml.

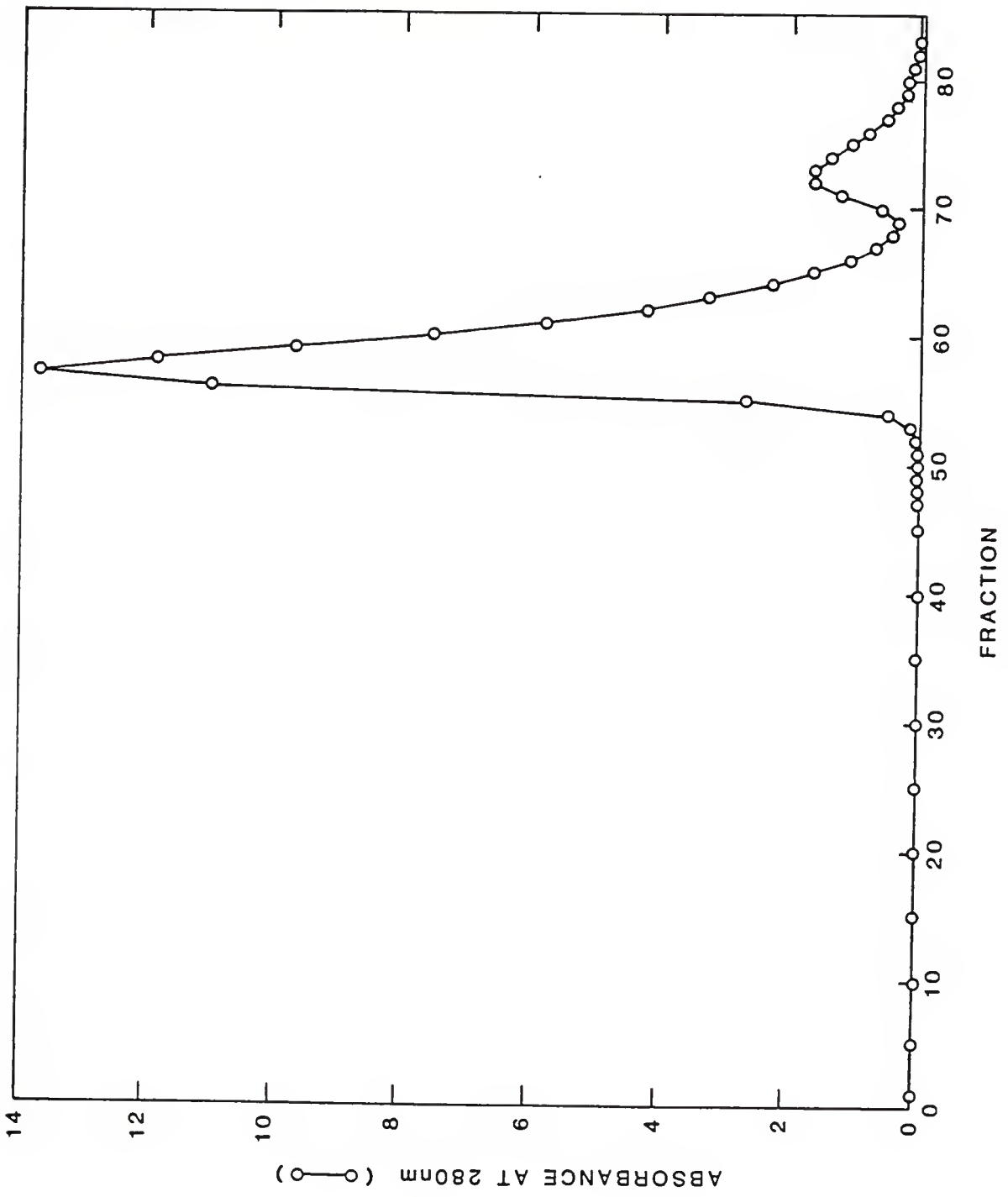
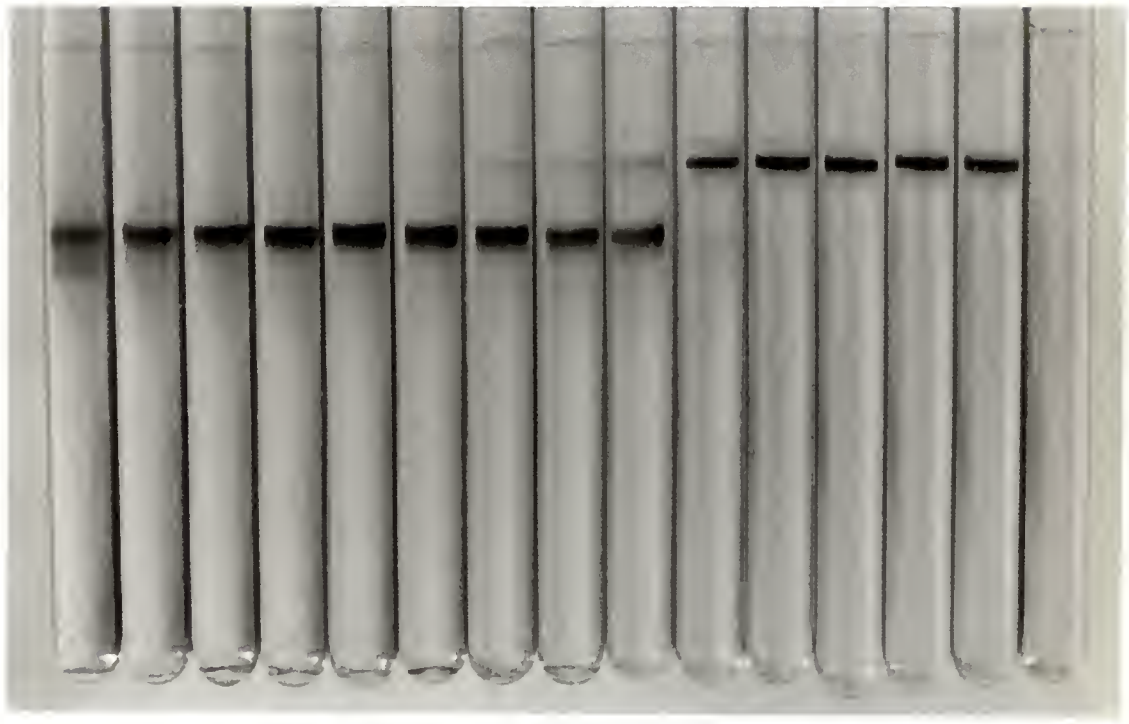

ABSORBANCE AT 280nm (o—o)
0
2
4
6
8
10
12
14
0
10
20
30
40
50
60
70
80
FRACTION

FIGURE 25

Polyacrylamide disc gel electrophoresis of successive protein fractions eluted from a Sephacryl S-200 column during chromatography of endoglucanase E.

Shown are gels of fractions 54-80 monitoring the separation of Endo E on Sephacryl S-200 (see Fig. 24). All gels were stained for protein with Coomassie Brilliant Blue and 40 µg of protein was applied to each gel. For further details, see "Results and Discussion: Endoglucanases".


54
55
56
58
60
62
64
66
68
70
72
74
76
78
80

eluted with a shallow pH 4.0-4.6 gradient; Endo B emerged first at pH 4.21 and Endo D at pH 4.37 (Fig. 26). Endo B (fractions 117-136) eluted with a minor low molecular weight contaminant and Endo D (fractions 142-158) eluted at high purity as determined by polyacrylamide disc gel electrophoresis (Fig 27). In later preparations, a low molecular weight contaminant that was present with Endo B, was removed by passage through a Sephacryl S-200 column under the same conditions as those under which Endo E was purified. A faint band noticeable just below the Endo D material on electrophoretic analysis, and also noticed in later preparations, was removed and may be due to some microheterogeneity of the Endo D. Purified Endo D was determined to have a specific activity of 161.4 $\Delta\emptyset_{sp}$/min/mg protein on carboxymethylcellulose and 24.7 µmoles glucose released/min/mg protein on Walseth cellulose.

The Endo B preparation which resulted from pH gradient elution of SP-Sephadex contained a minor contaminant which was removed by passage through a column of Sephadex G-75 (2.5 x 94 cm) equilibrated with 20mM sodium succinate with 3mM sodium azide at pH 5.0 (Fig 28). The purity of the Endo B material in fractions from this column was assessed by polyacrylamide disc gel electrophoresis (Fig. 29). Purified Endo B was determined to have a specific activity of 16.4 $\Delta\emptyset_{sp}$/min/mg protein on carboxymethylcellulose and 3.4 µmoles glucose released/min/mg protein on Walseth cellulose. Each of the pure endoglucanase proteins were determined to be glycosylated by staining the polyacrylamide disc gels for carbohydrate (Fig. 30).

FIGURE 26

Elution pattern from SP-Sephadex column chromatography of endoglucanase B and endoglucanase D using a pH gradient.

Conditions of elution are described in "Results and Discussion: Endoglucanases". The volume of each fraction was 5.25 ml.

ENDOGLUCANASE ACTIVITY [ΔΦsp/min/ml (□—□)
ABSORBANCE AT 280nm (○—○)
pH (△—△)
FRACTION
0
8
16
24
32
40
48
0
0.1
0.2
0.3
0.4
0.5
0.6
4.0
4.2
4.4
4.6
5
40
110
120
130
140
150
160
170
180

FIGURE 27

Polyacrylamide disc gel electrophoresis of successive protein fractions eluted from an SP-Sephadex column during chromatography of endoglucanase B and endoglucanase D using a pH gradient.

Shown are gels of fractions 114-166 monitoring the separation of Endo B and Endo D on SP-Sephadex (see Fig. 26). All gels were stained for protein with Coomassie Brilliant Blue. 40 µg of protein was applied to each gel and gel C was a control with no protein added. For further details, see "Results and Discussion: Endoglucanases".


C
114
117
120
123
126
129
132
135
138
141
144
147
150
154
158
162
166

FIGURE 28

Elution pattern from Sepphadex G-75 column chromatography of endoglucanase B.

Conditions of elution are described in "Results and Discussion: Endoglucanases". The volume of each fraction was 5.25 ml.

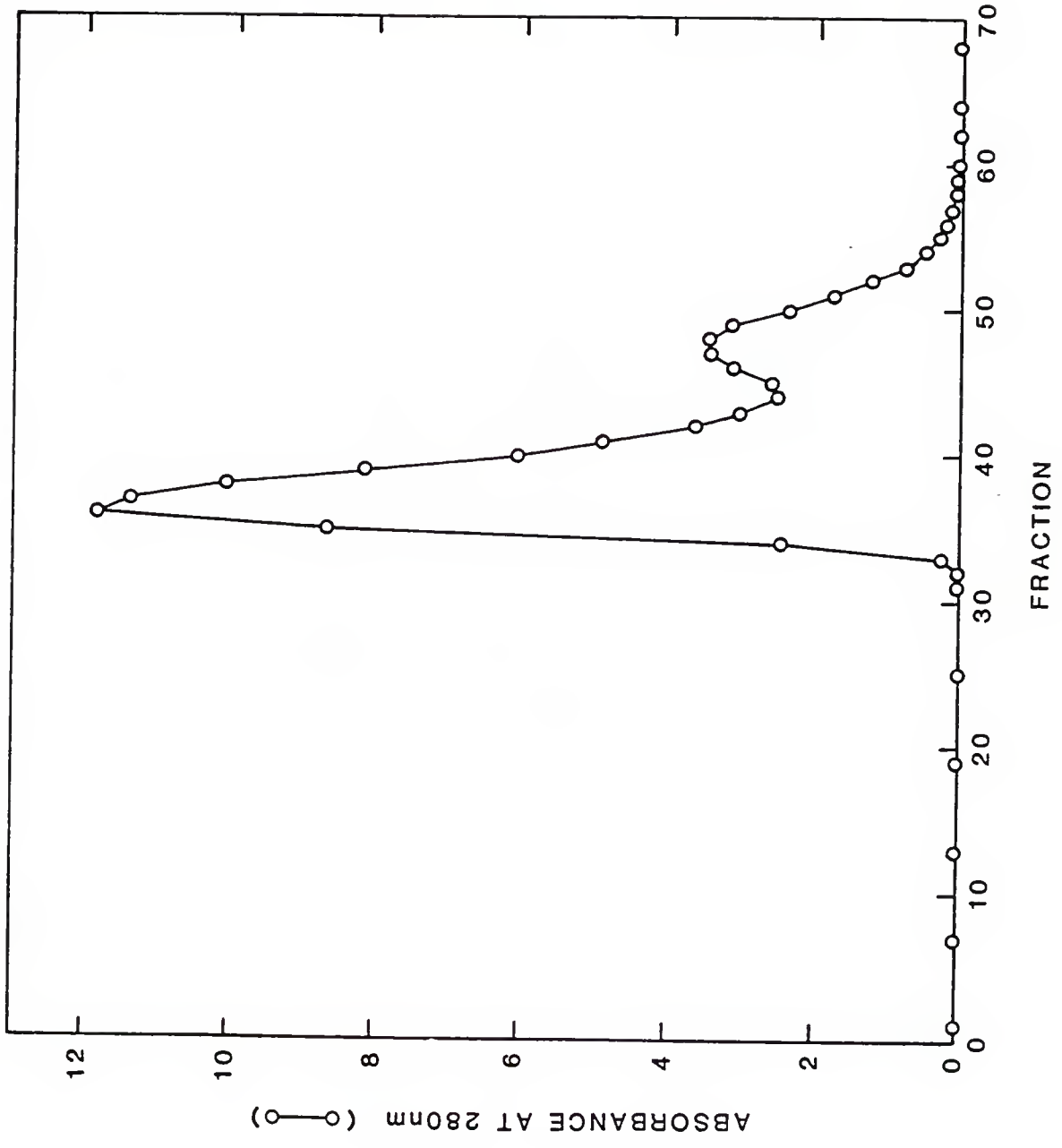

ABSORBANCE AT 280nm (o—o)
0
2
4
6
8
10
12
0
10
20
30
40
50
60
70
FRACTION

FIGURE 29

Polyacrylamide disc gel electrophoresis of successive protein fractions eluted from a Sephadex G-75 column during chromatography of endoglucanase B.

Shown are gels of fractions 34-51 monitoring the separation of Endo B on Sephadex G-75 (see Fig. 28). 40 µg of protein was applied to each gel and all were stained with Coomassie Brilliant Blue for protein. For further details, see "Results and Discussion: Endoglucanases".

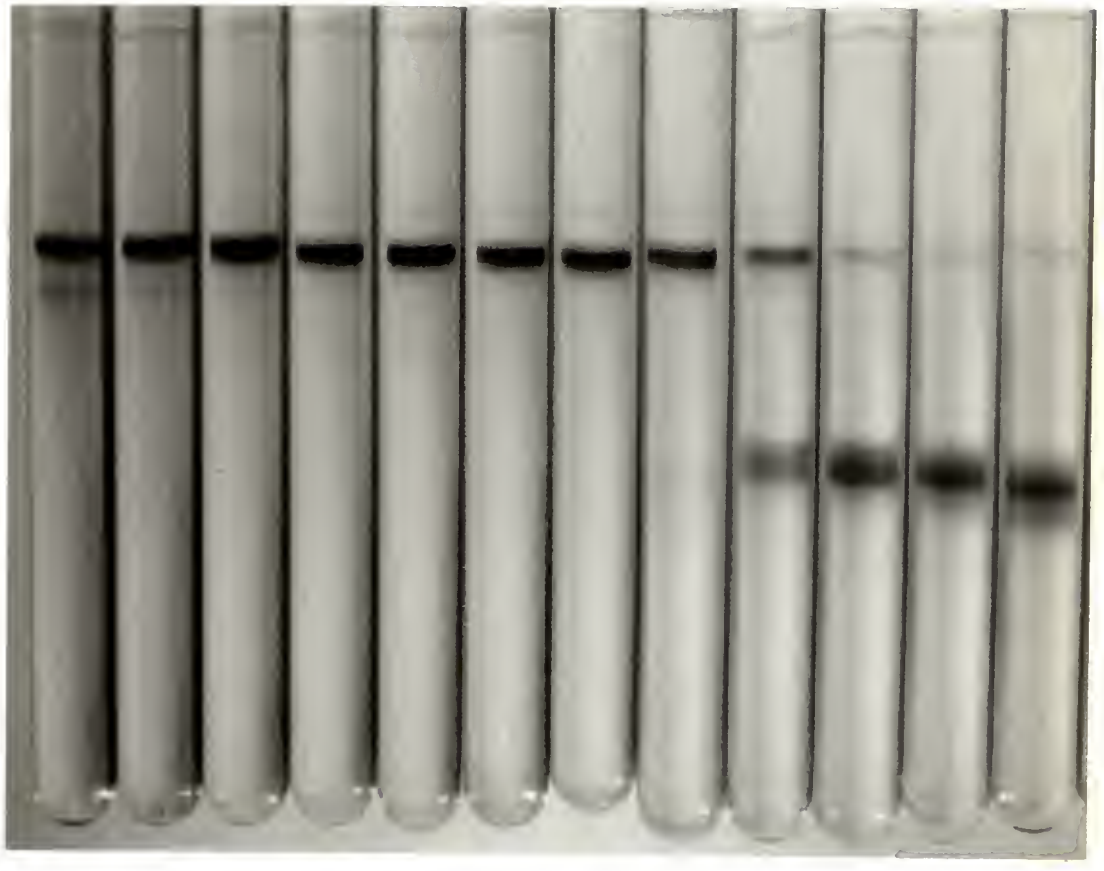

34
35
37
39
40
41
42
43
45
47
49
51

FIGURE 30

Polyacrylamide disc gel electrophoresis of crude extracellular protein prepared from T. reesei QM 9414 and purified endoglucanases.

Lanes 1 and 2 contain 100 µg each of crude extracellular protein from Trichoderma reesei QM 9414 grown on cellulose. Lanes 3 and 4 contain 30 µg each of purified Endo B. Lanes 5 and 6 contain 30 µg each of purified Endo D. Lanes 7 and 8 contain 30 µg each of purified Endo E. Lanes 1, 3, 5 and 7 were stained with Coomassie Brilliant Blue for protein and Lanes 2, 4, 6 and 8 were stained with the periodic acid-Schiff reagent for carbohydrate.


1 2 3 4 5 6 7 8

^{13}C-NMR--Proton decoupled ^{13}C-NMR spectra at 75.5 MHz were obtained for each of the endoglucanases in order to compare the types of oligosaccharides attached to them with those side chains previously characterized for CBH I(D) (Fig. 14,Panel A) and CBH II (Fig. 21,Panel A). As NMR provides information about the entire molecule being analyzed and is also a non-destructive technique, it was hoped that this procedure would reveal the structure of the carbohydrate by comparison, and thereby preserve material that would have been consumed during the chemical analysis. Pertinent signals from the ^{13}C-NMR analysis of the endoglucanases are listed in Table XII.

Endo B yielded two predominant signals in the anomeric region at 105.1 and 100.8 ppm, indicative of α-linked mannosyl and α-linked glucosyl residues, respectively (Fig. 31,Panel A). These could be assigned due to the extensive chemical characterization performed on the cellobiohydrolases. The signal at 100.8 ppm also contains another shoulder signal at 100.7 ppm, the origin of which has not yet been identified. Also in the anomeric region were three smaller signals at 101.5, 102.3 and 104.0 ppm. The first two of these have been assigned to mannosyl residues α-linked to serine and mannosyl residues α-linked to threonine, respectively,. These assignments are supported by reported values for anomeric signals of ManαThr and ManαSer standards of 102.8 and 101.8 ppm, respectively [100]. The signal at 104.0 ppm has not yet been identified and like that associated with CBH II, is thought to be an attachment to another as yet unidentified amino acid. The similarity of this spectra with that from CBH II would suggest that trisaccharides were the most predominant species of oligosaccharide covalently bound to Endo B and that these were

TABLE XII

SELECTED ^{13}C-NMR SIGNALS DUE TO CARBOHYDRATE COVALENTLY ATTACHED TO THE ENDOGLUCANASES OF *T. reesei* QM 9414

Proton coupled and decoupled ^{13}C-NMR spectra were performed at 75.5 MHz. 150 mg of each of the endoglucanases, B,D and E were used. Chemical shifts are expressed in PPM. For further details, see "Experimental Procedures".

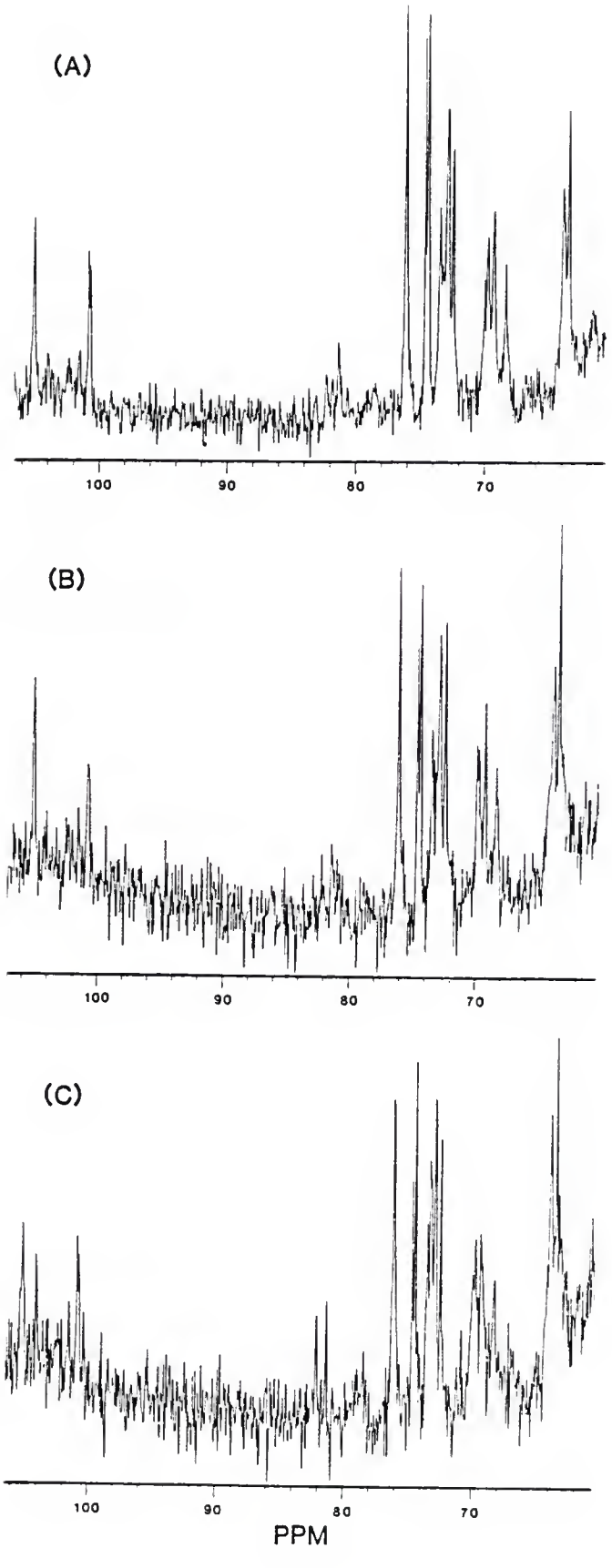
	Chemical Shifts Relative to TSP			
Carbon	ENDO B	ENDO D	ENDO E	Residue
Anomeric Carbons				
C-1	104.0	104.0	104.1	-
	102.3	102.3	102.3	Man
	101.5	101.5	101.5	Man
C-1'	105.1	105.1	105.1	Man
C-1"	100.8	100.8	100.8	Glc
C-1*	100.7	-	100.7	-
Hydroxymethyl Carbons				
C-6	63.5	63.4	63.5	Man
C-6"	63.9	63.8	63.9	Glc

* This signal has not yet been assigned.

FIGURE 31

Proton decoupled ^{13}C-NMR spectra of the endoglucanases purified from T. reesei QM 9414 grown on cellulose.

Spectra of (A) 150 mg of Endo B, (B) 150 mg of Endo D and (C) 150 mg of Endo E, were recorded at 75.46 MHz. Chemical shifts are reported in parts per million from internal TSP. Spectra were the result of (A) 38476 accumulations, (B) 41312 accumulations and (C) 41996 accumulations, respectively. For further details, see "Experimental Procedures".


(A)
100
90
80
70
(B)
100
90
80
70
(C)
100
90
80
70
PPM

attached to threonyl and seryl residues and perhaps some other as yet unidentified amino acid.

Endo B also gives rise to two predominant signals in the hydroxymethyl region (63-66 ppm) at 63.5 and 63.9 ppm, suggesting that the trisasccharide must contain one 6-substituted residue. The overall pattern of the signals in the carbohydrate region of the spectrum (60-110 ppm) was very similar to that found with the cellobiohydrolases which suggests that the oligosaccharides are also very similar.

Endo D yielded a spectrum very similar to that from Endo B (Fig. 31, Panel B). The main difference noticed in the signals is the absence of the split peak at 100.7 ppm for this enzyme. At great amplification (not shown) a shoulder is visible on the side of the peak at 100.8 and so the signal at 100.7 ppm may be present, but merely unresolved. The other predominant signal at 105.1 ppm is present as it is with Endo B and also the two cellobiohydrolases.

Endo D, similarly yields two predominant signals in the hydroxymethyl region at 63.4 and 63.8 ppm, suggesting that if a trisaccharide is present, then it must contain a 6-substituted residue.

Endo E also gives rise to two predominant anomeric signals at 105.1 and 100.8 ppm, which were attributed to α-linked mannose and α-linked glucose residues, respectively (Fig 31,Panel C), for reasons mentioned above. The latter signal also contained a small shoulder signal at 100.7 ppm, similar to that found with Endo B, and which has not yet been assigned. Endo E also generates three smaller signals in the anomeric region at 101.5, 102.3 and 104.1 ppm. The former two were

assumed to be due to mannosyl residues α-linked to serine and mannosyl residues α-linked to threonine, respectively. The origin of the signal at 104.1 ppm has not yet been identified. These results suggest that trisaccharides are the most predominant oligosaccharides attached to Endo E and that they are attached to seryl and threonyl residues and perhaps some other amino acid(s).

Endo E also gives rise to two predominant signals in the hydroxymethyl region at 63.4 and 63.9 ppm, suggesting that these trisaccharides contain one 6-substituted residue. The entire carbohydrate region of the spectra generated with Endo E is very similar to that found with the other endoglucanases and also with the cellobiohydrolases.

Assessment of endoglucanase oligosaccharide structure--The great similarity in the ^{13}C-NMR spectra of the endoglucanases with those of the cellobiohydrolases suggests that the oligosaccharides attached to these enzymes are much alike. For the endoglucanases, two major signals in the anomeric region, at 104.0 and 100.7 ppm, cannot be assigned. The signal at 104.0 ppm, which was also found associated with CBH II, may be due to an attachment to an unidentified amino acid. The signal at 100.7 ppm, however, may be due to the presence of a larger oligosaccharide, eg. a tetrasaccharide, attached to the protein or to some other structure reflecting microheterogeneity. Although NMR analysis is very useful for comparing oligosaccharides with those of predetermined structure, eg. those from the

cellobiohydrolases, without the careful chemical characterization, the NMR data alone would be inadequate.

SUMMARY

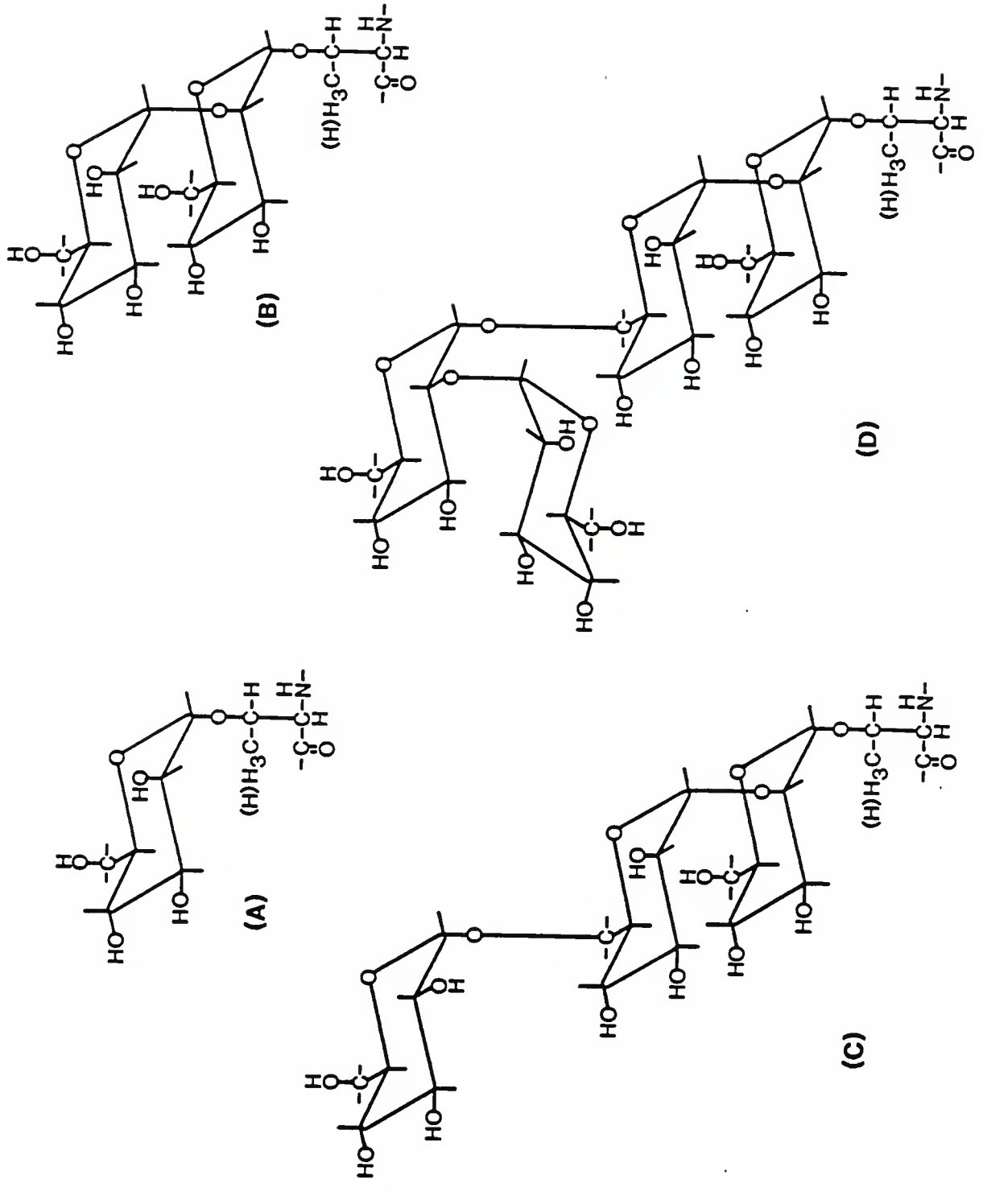
A series of unique and related oligosaccharides O-linked to two glycoprotein cellobiohydrolases, CBH I(D) and CBH II, from Trichoderma reesei QM 9414, have been purified and definitively characterized. The structures of these carbohydrates are shown diagrammatically in Fig. 32. From CBH I(D), which contained only 5.9% carbohydrate, were released 1.2 mono-, 1.1 di-, 4.2 tri- and 0.7 tetrasaccharides per protein molecule during reaction with alkaline borohydride. From CBH II, which contained 18.1% carbohydrate, reductive ß-elimination under the same conditions released 9.0 mono-, 1.6 di- and 14.9 trisaccharides per protein molecule.

A method was developed involving gas chromatographic analysis of mixed derivatives. With this method of sugar release from the glycoproteins by reductive ß-elimination, one can determine the total neutral carbohydrate composition, the nature of the sugar linking the oligosaccharide to the protein and the number of oligosaccharides O-linked to the protein. The sugars linking the side chains to the protein were reduced under the ß-elimination conditions used and thus were easily identified as the peracetylated alditols by gas chromatography; whereas other sugars in the side chains were not reduced and so were subsequently identified as the peracetylated aldononitriles. These analyses were carried out simultaneously requiring a single sample. This was an important part of the

FIGURE 32

Proposed structures of the oligosaccharides covalently attached to the glycoprotein cellulases from *T. reesei* QM 9414.

Shown are diagrammatic representations of the (A) monosaccharide (Man), (B) disaccharide (Manα(1-2)Man), (C) trisaccharide (Glcα(1-6)Manα(1-2)Man) and (D) tertrasaccharide (Manα(1-2)Glcα(1-6)Manα(1-2)Man).


(A)
(B)
(C)
(D)

glycoprotein characterization as it determined both the amount and the nature of the oligosaccharides attached to these proteins, and it is a method which should be widely applicable to any glycoproteins containing O-linked oligosaccharides. This analysis showed CBH I(D) to contain six or seven oligosaccharide chains linked through mannose to the protein and showed CBH II to contain 25 oligosaccharide chains each of which also was linked through a mannosyl residue to the protein. These values agreed very well with the respective values obtained for the number of oligosaccharides per molecule derived from HPLC analysis of the side chains released by reductive ß-elimination.

Methylation analysis provided information as to the types of linkages present in the oligosaccharides and the partially methylated alditol acetates produced were conclusively identified by gas chromatography/mass spectrometry. A comparison of the methylation patterns of the intact glycoproteins with those of the reduced oligosaccharides released from the glycoproteins by ß-elimination revealed that the mannosyl residues attached to the protein were each substituted in the 2-position. For the trisaccharide, the second sugar was substituted at the 6-position, although it could not be determined whether this was a mannosyl or a glucosyl residue. For the tetrasaccharide, the reducing end mannose was substituted in the 2-position, and the chain also contained a 6-substituted residue and another 2-substituted residue, although the order of these last two could not be determined solely by the GC/MS method. Acetolysis demonstrated that the (1-6) glycosidic linkage was in the middle of the tetrasaccharide and also confirmed that the central residue of the trisaccharide was substituted in the 6-position.

Sequential digestion of these carbohydrates with α-glucosidase and α-mannosidase determined that each sugar residue in the oligosaccharides were linked through α-glycosidic bonds. The sequence of residues and corresponding configurations in each chain was shown to be: ManαGlcαManαmannitol, GlcαManαmannitol and Manαmannitol, for the tetra-, tri- and disaccharides, respectively.

From these experiments, it was possible to define the structure of the oligosaccharides that were attached to the cellobiohydrolases (Fig. 32). These oligosaccharides are the only documented occurence of a Glcα(1-6)Man linkage as this author is not aware any published description of such a structure. Although the structures of the oligosaccharides attached to CBH I(C) from Trichoderma viride were not determined, Gum [93] did show them to be O-linked to the protein, to contain predominantly mannose and glucose with some (1-6) glycosidic linkages and also contain α-mannosidase sensitive mannose residues in the disaccharide.

NMR analyses of these glycoproteins and of the oligosaccharides released from them, also provided information about the carbohydrate structure. NMR has the advantage that it is non-destructive and so permits recovery of all the material after analysis; however it has the disadvantage that, compared to other analytical techniques, a substantial quantity of material (approximately 5 μmoles) is needed for analysis. ^{13}C-NMR spectra of CBH I(D) revealed three predominant signals in the anomeric region which were assigned to the anomeric carbons of the most abundant side chain, the trisaccharide. Most important was the strength of the signal at 102.5 ppm as this indicated that the oligosaccharides were attached predominantly to

threonyl residues of the protein. This hypothesis was supported by the published spectra of ManαThr and ManαSer standards [100] and also by the established polypeptide sequence analysis [60,61] which had determined that the carbohydrate was attached within a short region containing seven or eight threonyl residues near the C-terminus of CBH I. This specific linkage was clearly indicated by amino acid analysis of α-amino butyric acid residues formed following ß-elimination, $PdCl_2$ reduction and subsequent hydrolysis of the protein. This established that there were 7 oligosaccharide attachment sites to threonyl residues on the protein. CBH II, on the other hand, showed only two predominant signals in the anomeric region by ^{13}C-NMR analysis and the anomeric signal corresponding to the sugar attached to the protein was instead replaced by a number of smaller signals. Two of these were identified as being due to mannosyl residues α-linked to seryl and to threonyl residues on the protein and the other was not identified and was thought to represent an attachment to some other amino acid. Hydroxyproline and hydroxylysine were obvious candidates, but neither was found to be present after amino acid analysis of CBH II. Unfortunately, very little information about the ^{13}C-NMR analysis of glycoproteins with O-linked oligosaccharides is available for comparison at this time.

From studies on glucoamylase from Aspergillus niger, Dill and Allerhand [102] have assigned several of the natural abundance ^{13}C-NMR signals to specific carbons on the oligosaccharide chains. Unfortunately, the glycoprotein preparation analyzed was a mixture of two isozymes and assignments were ambiguous due to incomplete structural characterization of the carbohydrate. These authors

assigned a signal at 103.6 ppm to that of α-D-mannopyranosyl residues (unsubstituted at carbon 2) which were involved in glycosidic linkages to carbon 2 or carbon 3 of adjacent α-D-mannopyranosyl residues. This work assigns a signal at 105.1 ppm to these residues. A signal at 102.5 ppm was assigned to that of α-D-mannopyranosyl residues (substituted at carbon 2) involved in anomeric linkages to carbon 2 or carbon 3 of adjacent α-D-mannopyranosyl residues. This work assigned a signal at 102.5 ppm to similar residues attached to threonine on the polypeptide. Dill and Allerhand also assigned signals at 101.8, 101.1 and 100.8 ppm to α-D-mannopyranosyl residues attached to threonyl or seryl residues on the polypeptide or carbon 6 adjacent mannosyl or glucosyl residues. This work has assigned a signal at 101.5 ppm to mannosyl residues α-linked to serine on the polypeptide and a signal at 100.8 ppm to glucosyl residues α-linked to carbon 6 of adjacent mannosyl residues.

Determination of the coupling constants of anomeric carbons by coupled ^{13}C-NMR analysis showed that all of the sugar residues attached to the two proteins were in the α-configuration. Other important information derived from the ^{13}C-NMR spectra was the indication of the number of hydroxymethyl carbons present. It should be mentioned that the C-6 of a free hexose residue is a hydroxymethyl group and that the signal it generates is shifted when the sugar residue is sustituted at this position. Thus the number of hydroxymethyl carbon signals can provide information about the number of 6-substituted residues. The trisaccharides from both cellobiohydrolases were found to have one 6-substituted residue and

the disaccharide from CBH I(D) was found to have none, which is consistent with the chemical evidence.

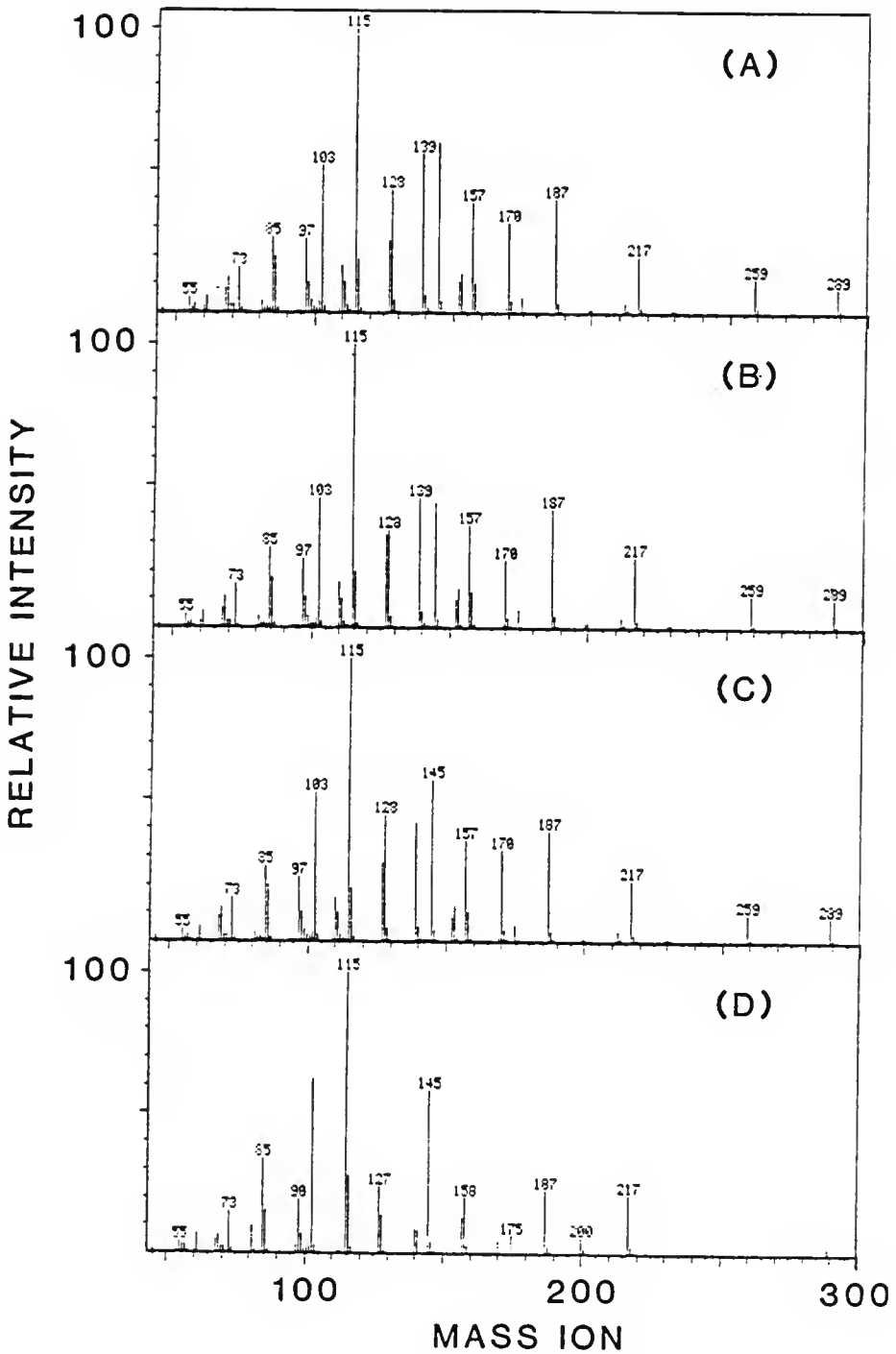
Three endoglucanases, B, D and E, were purified and although it is not known how these are related, comparison of the ^{13}C-NMR spectra with those of the cellobiohydrolases has determined that the arrays of oligosaccharides attached are very similar, if not identical. It should be pointed out that without definitive chemical characterization of the oligosaccharides attached to these glycoproteins, the complex NMR spectra permit only limited interpretation. With this information available, however, the carbohydrate of unknown samples can be compared with glycoproteins of known structure, thus saving considerable time and material.

It can be concluded that each of the major glycoenzymes secreted by Trichoderma reesei QM 9414 grown on cellulose are glycosylated with a series of unique and related oligosaccharides. Now that this has been determined, the role that this carbohydrate plays, be it in catalysis, secretion, stability, subsite affinity, biosynthesis or microheterogeneity, can now be elucidated.

APPENDIX A

ELECTRON IMPACT MASS SPECTRA OF PERACETYLATED ALDITOLS.

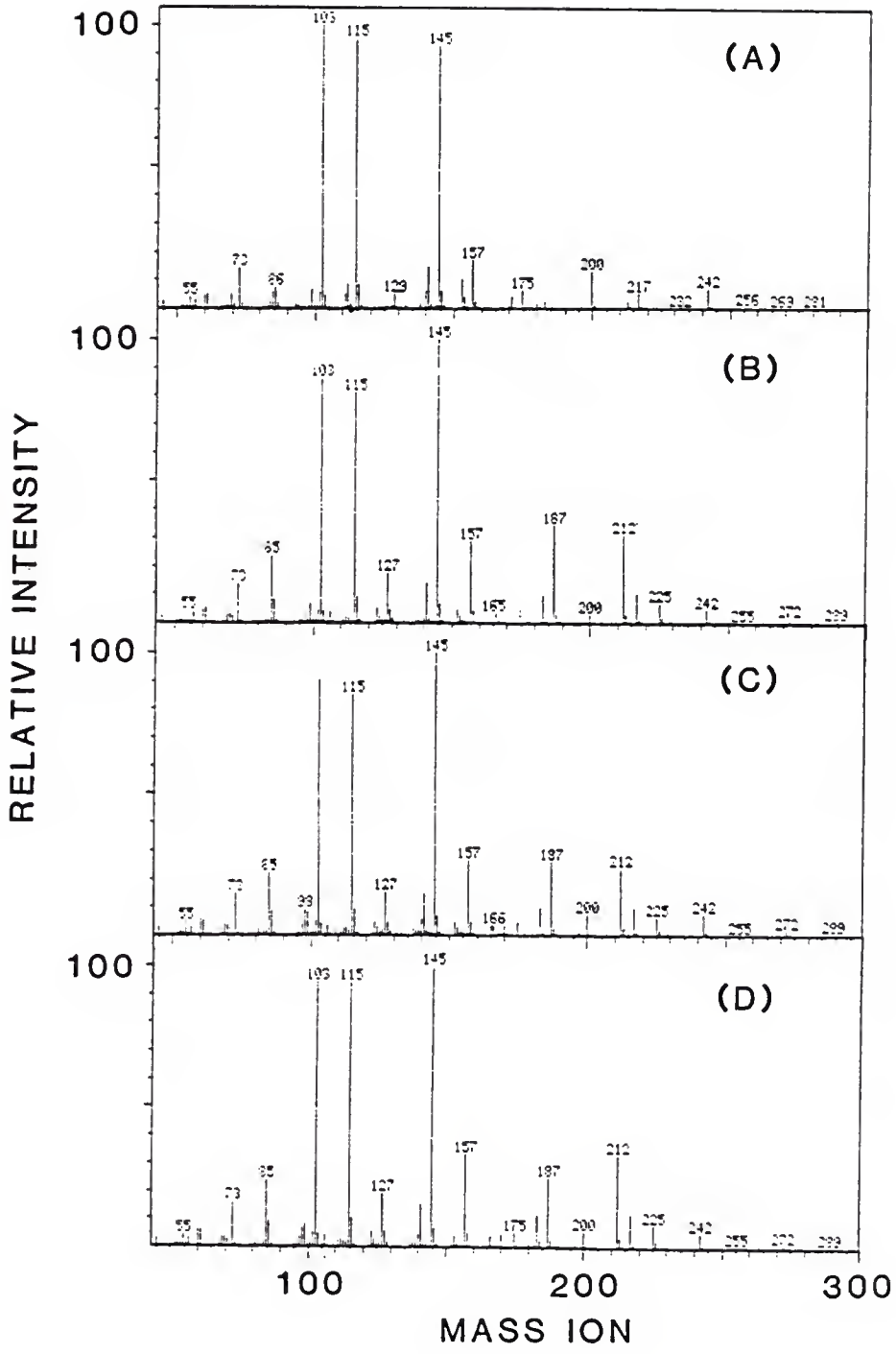
Shown are the spectra of peracetylated (A) mannitol, (B) galactitol, (C) glucitol and (D) xylitol. For further details, see "Experimental Procedures".


100
(A)
115
103
139
123
157
187
178
85
97
73
217
259
289
100
(B)
115
103
139
123
157
187
85
97
73
178
217
259
289
100
(C)
115
103
145
123
157
187
85
97
73
178
217
259
289
100
(D)
115
145
85
73
98
127
158
187
217
175
200
RELATIVE INTENSITY
100
200
300
MASS ION

APPENDIX B

ELECTRON IMPACT MASS SPECTRA OF PERACETYLATED ALDONONITRILES.

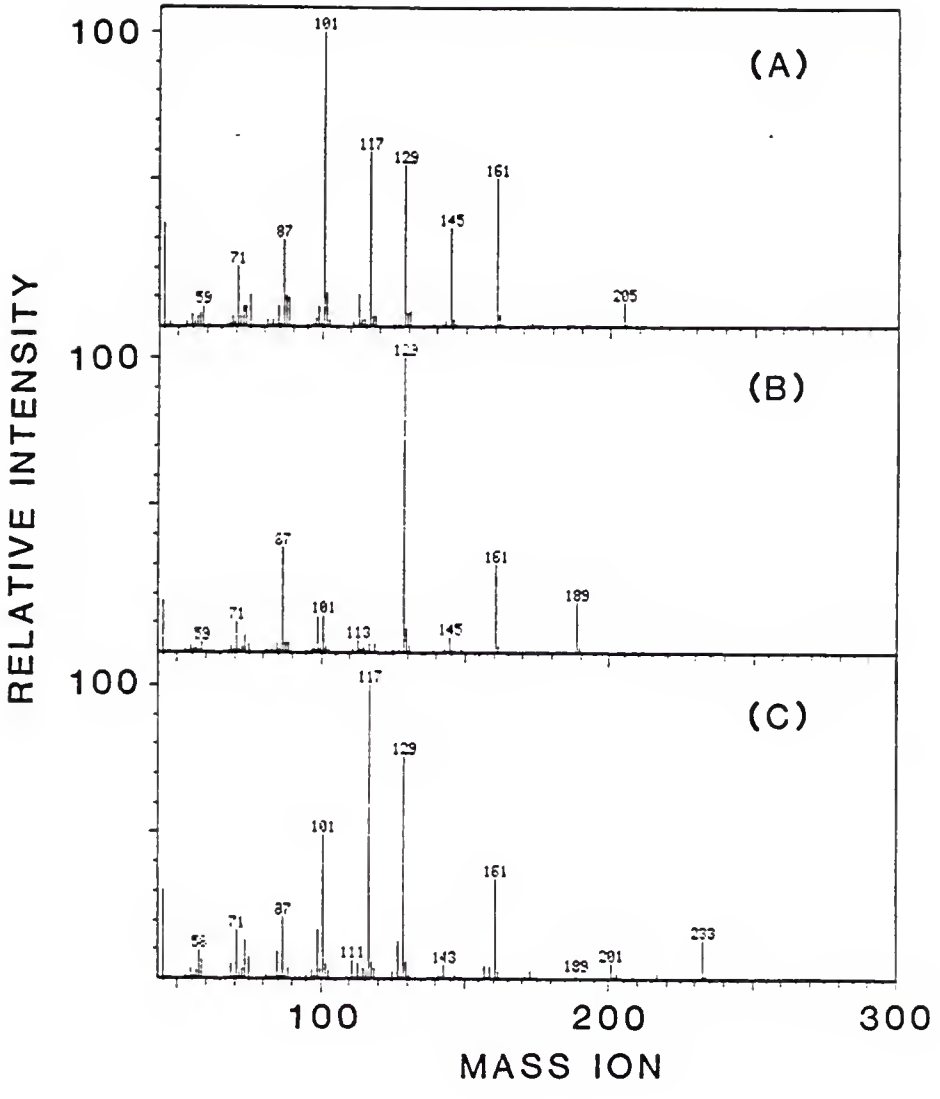
Shown are the spectra of peracetylated (A) xylononitrile, (B) mannononitrile, (C) gluconononitrile and (D) galactononitrile. For further details, see "Experimental Procedures".

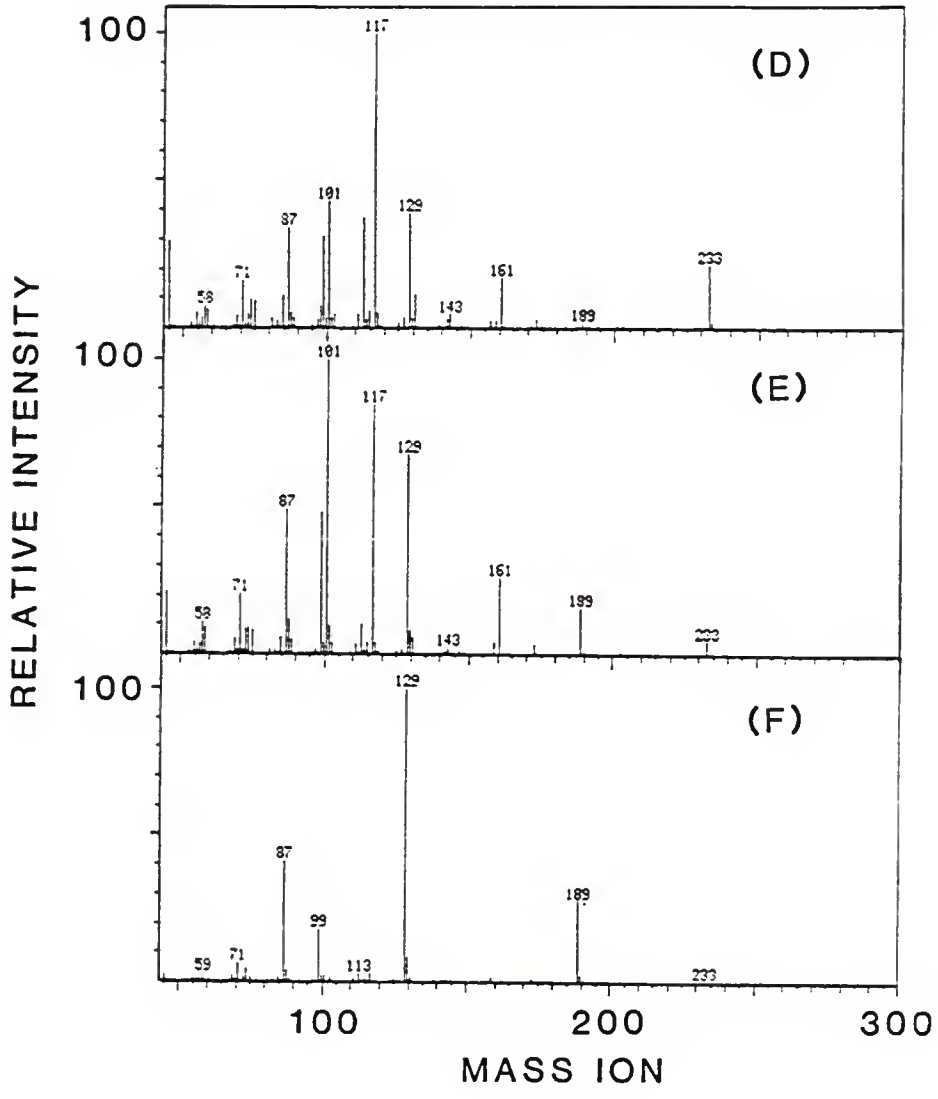

100
(A)
103
115
145
73
85
157
200
217
242
100
(B)
145
103
115
65
73
127
157
187
212
100
(C)
145
115
65
73
127
157
187
212
100
(D)
103
115
145
73
85
127
157
187
212
RELATIVE INTENSITY
100
200
300
MASS ION

APPENDIX C

ELECTRON IMPACT MASS SPECTRA OF PARTIALLY METHYLATED ALDITOL ACETATES.

Standards were generated by methylation of yeast mannan. Shown are the spectra of (A) 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl mannitol, (B) 1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl mannitol, (C) 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl mannitol, (D) 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl mannitol, (E) 1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl mannitol and (F) 1,2,5,6-tetra-O-acetyl-3,4-di-O-methyl mannitol. For futher details, see "Experimental Procedures".

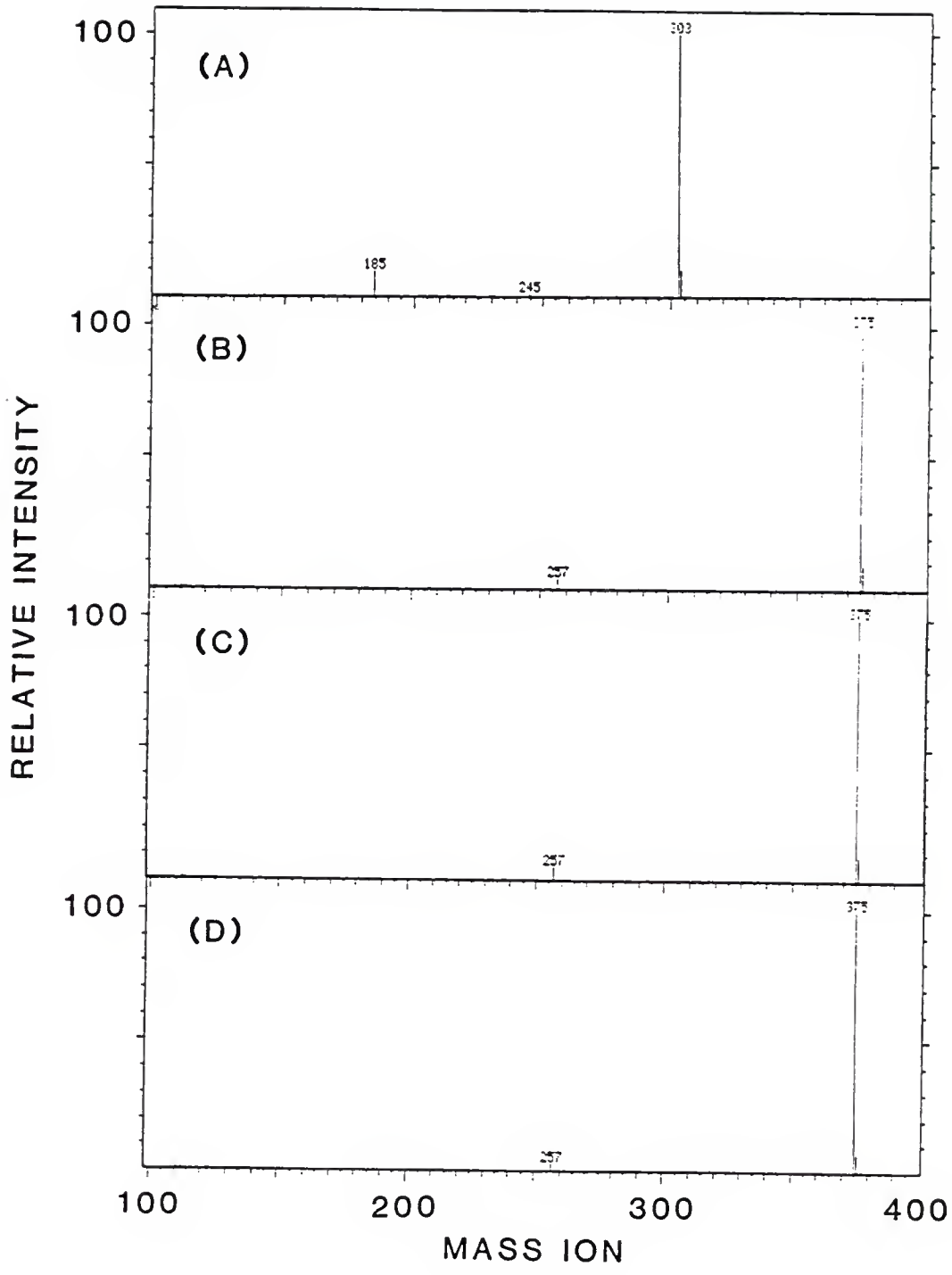

100
101
(A)
117
129
161
145
87
71
59
205
100
(B)
87
161
189
71
101
59
113
145
100
117
(C)
129
101
161
87
71
111
143
189
201
233
100
200
300
MASS ION
RELATIVE INTENSITY


100
(D)
117
101
129
87
71
161
233
58
143
189
100
(E)
101
117
129
87
71
161
189
58
143
233
100
(F)
129
87
189
99
59
71
113
233
RELATIVE INTENSITY
100
200
300
MASS ION

APPENDIX D

CHEMICAL IONIZATION MASS SPECTRA OF PERACETYLATED ALDITOLS.

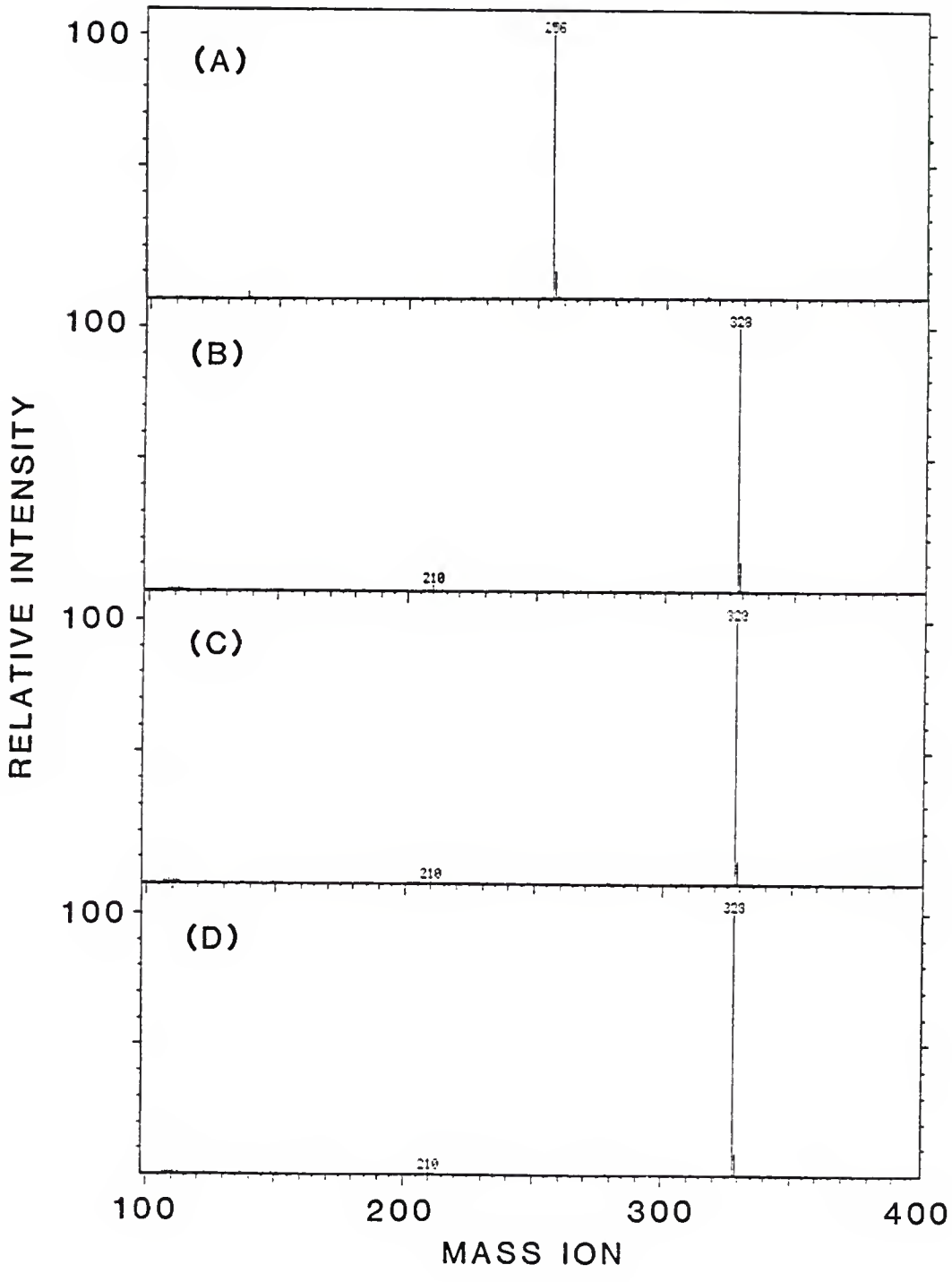
Shown are the spectra of peracetylated (A) xylitol, (B) mannitol, (C) galactitol and (D) glucitol. For further details, see "Experimental Procedures".


100
(A)
303
185
245
100
(B)
257
100
(C)
257
100
(D)
257
RELATIVE INTENSITY
100
200
300
400
MASS ION

APPENDIX E

CHEMICAL IONIZATION MASS SPECTRA OF PERACETYLATED ALDONONITRILES.

Shown are the spectra of peracetylated (A) xylononitrile, (B) mannononitrile, (C) gluconononitrile and (D) galactononitrile. For further details, see "Experimental Procedures".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(A)
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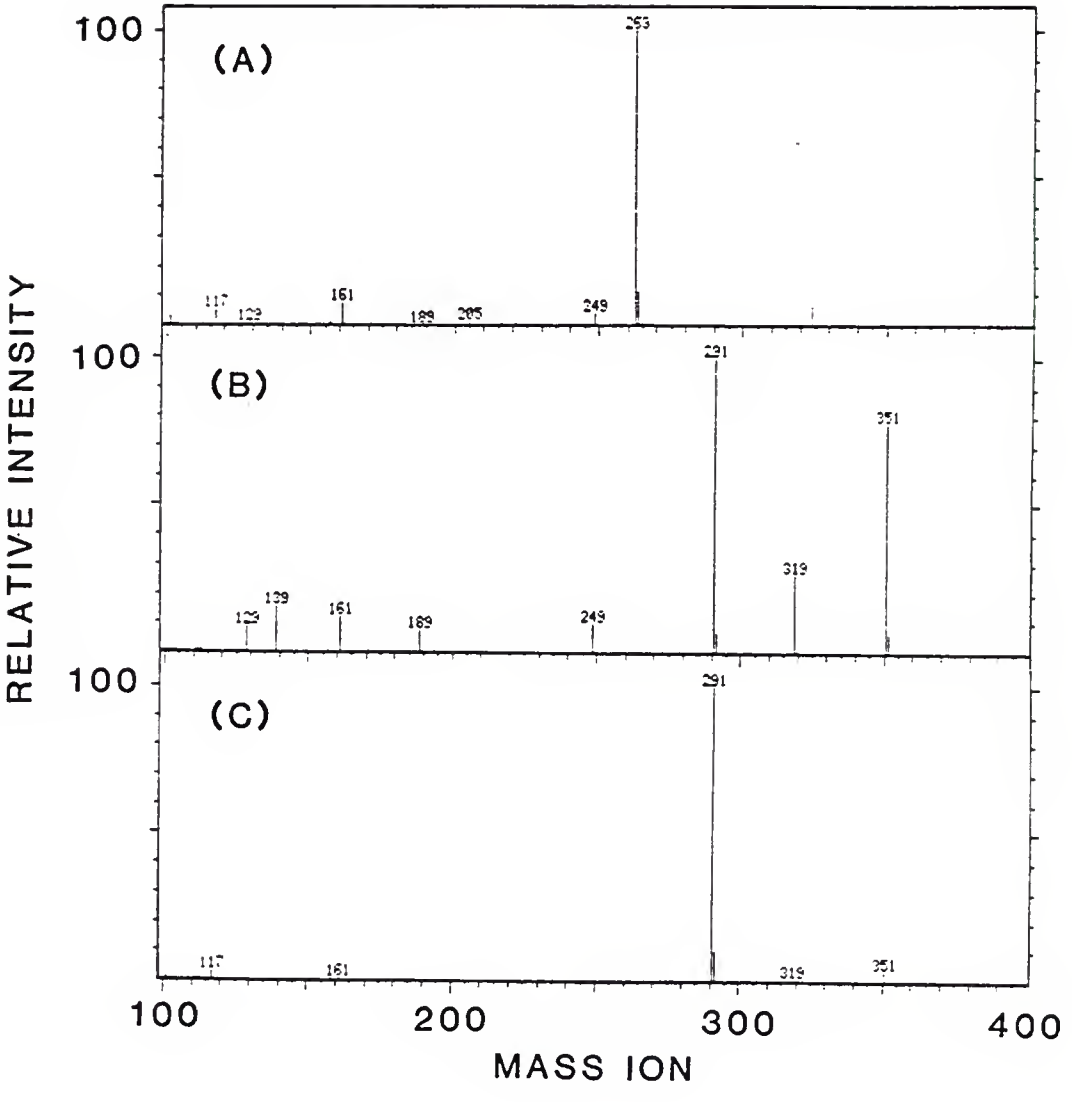
100
(B)
329
210
100
(C)
329
210
100
(D)
329
210
RELATIVE INTENSITY
100
200
300
400
MASS ION

APPENDIX F

CHEMICAL IONIZATION MASS SPECTRA OF PARTIALLY METHYLATED ALDITOL ACETATES.

Standards were generated by methylation of yeast mannan. Shown are the spectra of (A) 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl mannitol, (B) 1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl mannitol, (C) 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl mannitol, (D) 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl mannitol, (E) 1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl mannitol and (F) 1,2,5,6-tetra-O-acetyl-3,4-di-O-methyl mannitol. For further details, see "Experimental Procedures".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(B)
291
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319
123
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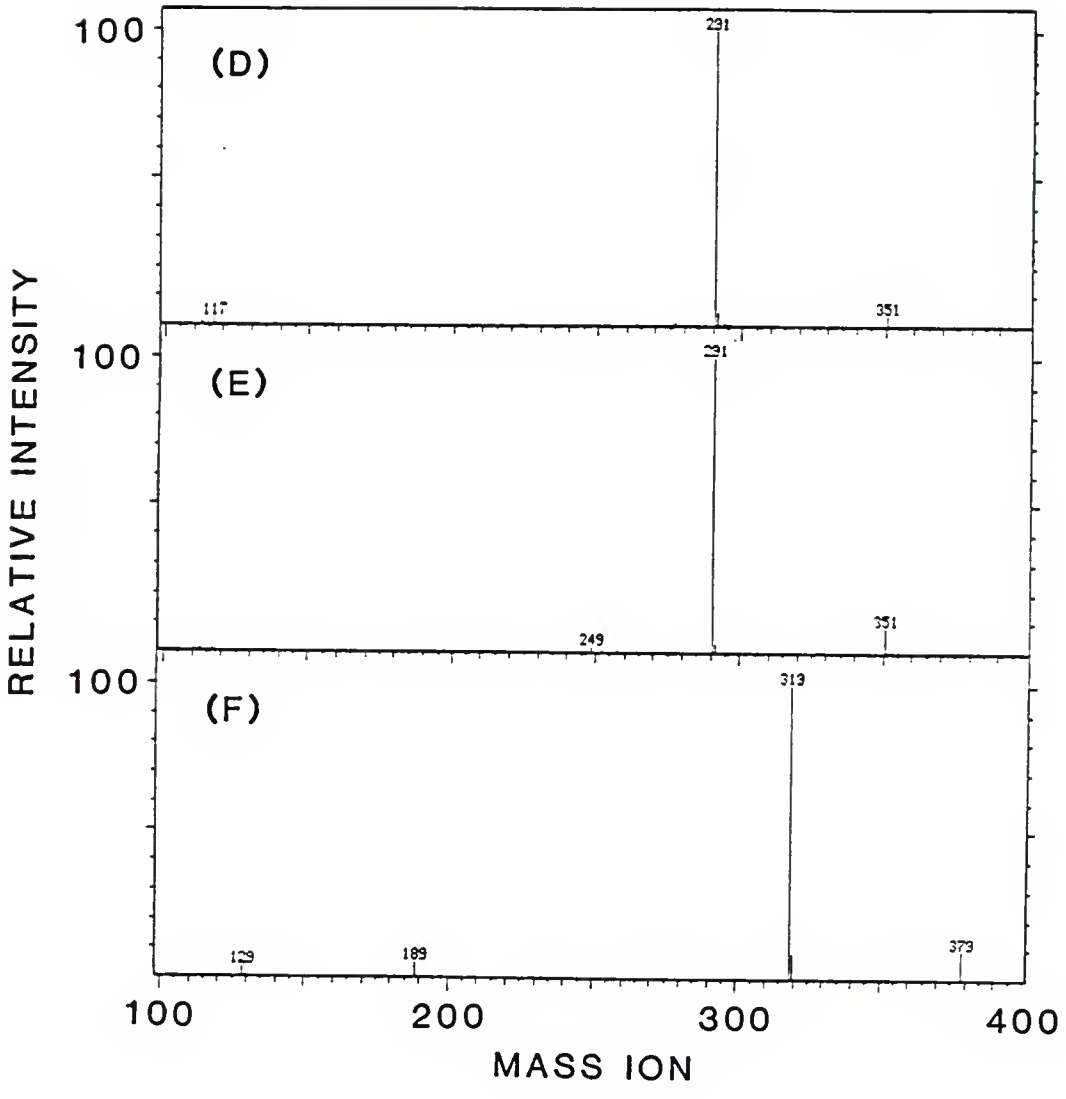
249
100
(C)
291
117
161
319
351
RELATIVE INTENSITY
100
200
300
400
MASS ION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(D)
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117
351
100
(E)
231
249
351
100
(F)
313
129
189
379
RELATIVE INTENSITY
100
200
300
400
MASS ION

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BIOGRAPHICAL SKETCH

Charles P. du Mée was born in Curepipe, Mauritius, on November 10, 1955. After five years in Mauritius and ten years in England, he moved to the United States in 1970. The author received his high school diploma from Gaithersburg High School, Maryland, in 1973, graduating a year early and then attended Montgomery College, Maryland, for one and a half years. He was then awarded a World Bank Scholarship and in 1975, he left to study chemistry at the University of Natal in the Republic of South Africa. He spent four years there, receiving his B.Sc. degree in chemistry and biochemistry in 1977 and his B.Sc.Hons. degree in biochemistry in 1978. While there, he captained the University of Natal Basketball Club and received his University Colors in the sport in 1977.

He then returned to the U.S. where he worked as a research assistant in a biochemistry laboratory at the University of Maryland. After a brief tenure, he was admitted to graduate school at Virginia Polytechnic Institute and State University in 1979, at which time he began his research with his advisor, Dr. Ross D. Brown, Jr. In 1980, he transferred with his advisor to the University of Florida where he completed his studies. The author married his wife, Linda, in 1979 before starting graduate school and they have two children, Colin and Ian, who were both born during his graduate career.

He is a member of the Division of Microbial and Biochemical Technology, the Division of Carbohydrate Chemistry and the Cellulose, Paper and Textile Division of the American Chemical Society. He is also a member of the Society of Complex Carbohydrates.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1984

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